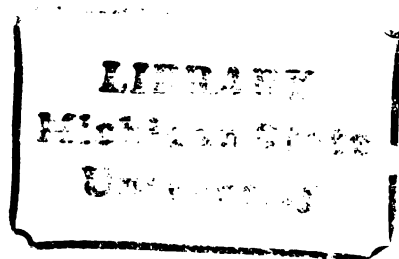




101
610
THS



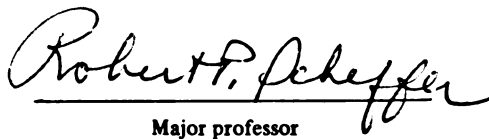
This is to certify that the

thesis entitled
EFFECTS OF HOST-SPECIFIC TOXIN FROM
ALTERNARIA ALTERNATA F.SP. LYCOPERSICI
ON SUSPENSION CULTURES OF LYCOPERSICON
ESCULENTUM

presented by
Sara-Ellen Barsel

has been accepted towards fulfillment
of the requirements for

M.S. degree in Plant Pathology


Major professor

Date Aug. 11, 1981



3 1293 10405 1697



299
~~KJ10~~
~~10405~~

OVERDUE FINES:

25¢ per day per item

RETURNING LIBRARY MATERIALS:

Place in book return to remove
charge from circulation records

EFFECTS OF HOST-SPECIFIC TOXIN FROM ALTERNARIA ALTERNATA F. SP.
LYCOPERSICI ON SUSPENSION CULTURES OF LYCOPERSICON ESCULENTUM

By

Sara-Ellen Barsel

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1981

ABSTRACT

EFFECTS OF HOST-SPECIFIC TOXIN FROM ALTERNARIA ALTERNATA F. SP. LYCOPERSICI ON SUSPENSION CULTURES OF LYCOPERSICON ESCULENTUM

By

Sara-Ellen Barsel

Host-specific toxin from Alternaria alternata f. sp. lycopersici was tested for effects in cell cultures of Lycopersicon esculentum. In leaflet bioassays, resistant cultivars tolerated 1000-fold higher concentrations of partially-purified toxin than did susceptible. Toxin was added to suspension cultures of resistant and susceptible cultivars at 0.2 - 4000 µg/ml. Resistant (cvs. Walter, Ace) and susceptible (cvs. Earlypak-7, VFN-Bush) genotypes, plus Nicotiana tabacum Wisconsin 38 were examined. Toxin effects on culture growth and mortality were assayed. The degree of toxin-induced growth inhibition and mortality was significantly greater in susceptible than in resistant genotypes. Discoloration of suspension cultures and accumulation of phenols increased in susceptible genotypes in proportion to the toxin concentration. Eleven fluorescent substances increased in filtrates of resistant cv. Walter with increases in the toxin concentration. Cell culture selections for toxin resistance are feasible in this system, because genotype-specificity is expressed in suspension culture.

This thesis is dedicated to
Hertha Karl, Patricia Bonamo,
Rosalind Franklin, and Walt Whitman.
Now I understand.

ACKNOWLEDGEMENTS

I would like to acknowledge the assistance provided by my committee, Dr. R. P. Scheffer, chairman, and Drs. N. Good and P. Carlson. I am especially grateful to Dr. Carlson for providing technical, financial and emotional support, in addition to the generous use of his laboratory. I am also indebted to Dr. K. Poff for his patient instruction and assistance in spectrophotometry and the generous use of his equipment. I am grateful to Dr. R. Hammerschmidt, who freely shared his enthusiasm for and knowledge of plant secondary metabolites. Of the many graduate students with whom I shared discussions, I would particularly like to thank Charles Caldwell for his instruction in column chromatography, and John Hunsperger for his instruction in horticultural practices and tissue culture techniques.

I would like to acknowledge the financial support of the U.S.D.A. and the Michigan A.E.S., in addition to a teaching assistantship from the Department of Botany and Plant Pathology.

TABLE OF CONTENTS

	Page
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
INTRODUCTION	1
LITERATURE REVIEW	3
Methods and limitations of traditional plant breeding	3
Applications of cell culture techniques to plant breeding	5
Recovery of variants from cell cultures without selection pressure	5
Traits expressed in intact tissue and <u>in vitro</u>	11
Selections for biochemical resistance in cell cultures	13
Selections for resistance to nonspecific toxins in cell cultures	13
Selections for resistance to host-specific toxins in cell cultures	16
Host-specific toxin-producing pathotypes of <u>Alternaria</u> <u>alternata</u>	18
Limitations of cell culture studies	24
MATERIALS AND METHODS	27
Tissue culture media	27
Cell culture initiation and maintenance	28
Fungal cultures	29
Toxin preparation	30
Toxin bioassay	31
Preparation of suspension cultures for toxin treatment	32
Growth determinations	32
Settled cell volume	32
Dry weight	32
Cell death	33
Preparation of samples for spectroscopy and chromatography ...	33
Thin layer chromatography	33
Paper chromatography	34
Spectroscopy	34

	Page
RESULTS	35
Evaluation of toxin preparation	35
Comparison of growth measurements for cell suspension cultures	36
Effects of toxin on growth of cell suspension cultures	38
Mortality of toxin-treated and control cells	58
Toxin-induced discoloration of suspension cultures	63
Spectrophotometric and chromatographic examination of filtrates of toxin-treated suspension cultures	64
DISCUSSION	73
BIBLIOGRAPHY	81

LIST OF TABLES

Table	Page
1 Growth rates and doublings of suspension cultures per day determined by settled cell volumes and dry weights of cells	37
2 Growth rates and doublings of suspension cultures per day determined by settled cell volumes	40
3 Effect of toxin on growth and mortality of suspension cultures of susceptible cv. VFN-Bush	43
4 Effect of toxin on growth and mortality of suspension cultures of resistant cv. Walter	46
5 Effect of toxin on growth and mortality of suspension cultures of resistant cv. Ace	49
6 Effect of toxin on growth and mortality of suspension cultures of susceptible cv. Earlypak-7	52
7 Summary of one-tailed t-tests of growth rates of suspension cultures of resistant cv. Walter and susceptible cv. VFN-Bush	53
8 Summary of one-tailed t-tests of growth rates of suspension cultures of resistant cv. Walter and susceptible cv. Earlypak-7	54
9 Summary of one-tailed t-tests of growth rates of suspension cultures of resistant cv. Ace and susceptible cv. VFN-Bush	55
10 Summary of one-tailed t-tests of growth rates of suspension cultures of resistant cv. Ace and susceptible cv. Earlypak-7	56

LIST OF FIGURES

Figure	Page
1 Effect of toxin (0, 4, 40, 400 $\mu\text{g/ml}$) on growth of cell cultures of susceptible tomato cv. VFN-Bush, as determined by regression analyses of settled cell volume data	42
2 Effect of toxin (0, 4, 40, 400 $\mu\text{g/ml}$) on growth of cell cultures of resistant cv. tomato Walter, as determined by regression analyses of settled cell volume data	45
3 Effect of toxin (0, 20, 200 2000 $\mu\text{g/ml}$) on growth of cell cultures of resistant cv. tomato Ace, as determined by regression analyses of settled cell volume data	48
4 Effect of toxin (0, 40 400, 4000 $\mu\text{g/ml}$) on growth of cell cultures of susceptible tomato cv. Earlypak-7, as determined by regression analyses of settled cell volume data	51
5 Percentage of toxin-induced cell death in suspension cultures of resistant cv. Walter and susceptible cv. Earlypak-7	60
6 Percentage of toxin-induced cell death in suspension cultures of resistant cv. Ace and susceptible cv. VFN-Bush	62
7 UV-absorption spectra of filtrates from cultures of resistant cv. Walter which had been treated with 40, 400, or 4000 μg toxin/ml	66
8 UV-absorption spectrum of aqueous toxin preparation (20 $\mu\text{g/ml}$, pH 6.0)	67
9 UV-absorption spectra of methanolic extracts from cultures of resistant cv. Walter which had been treated with toxin at 0, 0.4, 4, 40, 400, or 4000 $\mu\text{g/ml}$	69
10 UV-absorption spectra of methanolic extracts from cultures of susceptible cv. Earlypak-7 which had been treated with toxin at 0, 40, 400, or 4000 $\mu\text{g/ml}$	70

LIST OF ABBREVIATIONS

cv.	cultivar
ssp.	subspecies
TMS	Texas male-sterile
N	normal (cytoplasm)
mtDNA	mitochondrial deoxyribonucleic acid
cyclic-AMP	cyclic adenosine monophosphate
F ₁	first filial generation
F ₂	second filial generation
F ₃	third filial generation
MSO	methionine sulfoximine
NADH	reduced nicotinamide adenine dinucleotide
EDTA	disodium ethylenediaminetetraacetic acid
SH	sulphydryl
AM	<u>Alternaria mali</u> (in reference to host-selective toxin)
AK	<u>Alternaria kikuchiana</u> (in reference to host-selective toxin)
VFN	<u>Verticillium-Fusarium</u> -nematode-resistant tomato cultivars
UV	ultraviolet
rpm	revolutions per minute
log	logarithmic
g	gram
mg	milligram
µg	microgram
ng	nanogram
ml	milliliter
µl	microliter
nm	nanometer
µ	micron
p	para
v	volume
mM	millimolar

INTRODUCTION

Cell culture techniques developed within the last ten years have provided methods for the generation of variability, the selection of mutations, and the regeneration of plants from protoplasts and cells in culture. Application of biochemical selection pressure to cell cultures to increase the recovery of variants in a particular trait has become routine. One of the more successful selection methods involves the use of nonspecific and host-specific toxins as selective agents. This thesis describes an initial effort to use host-specific toxin as a selective agent in plant cell suspension cultures to obtain toxin-tolerant or toxin-resistant plants.

My study was concerned with the effects of the host-specific toxin produced by Alternaria alternata f. sp. lycopersici on suspension cultures of resistant and susceptible genotypes of Lycopersicon esculentum (tomato). A. alternata f. sp. lycopersici is the causal agent of stem canker disease of certain cultivars of fresh market tomatoes. Host-specific toxins have been isolated from culture filtrates of pathogenic isolates of the fungus (56,69,70,71), and shown to induce symptoms similar to those of natural infection on susceptible tomato cultivars. Tomato cultivars have been classified as resistant or susceptible with respect to the pathogen. I have examined the response of cells in vitro to the toxin, to determine whether sensitivity and insensitivity to toxin is correlated with the intact plant response to

the pathogen and its toxins. Hopefully, the work may also give a clearer understanding of the physiological responses of the host to the toxins. Successful demonstration that genotype-specific differences are expressed in culture, and are correlated with intact plant responses, would indicate that selection and recovery of toxin-tolerant or toxin-resistant variants is feasible in this system.

LITERATURE REVIEW

Methods and limitations of traditional plant breeding

The art of plant breeding has been practiced since plant domestication began, but emphasis has shifted from domestication of crop plant species to domestication of genes (40). With the application of Mendel's laws of inheritance to plant breeding, the initial focus was on selection of genes determining qualitative characteristics such as color, disease resistance, and gross morphological alterations (40). Attention has now shifted to inheritance of genes determining quantitative traits including yield, height, maturity (40), plant architecture, stress tolerance, and disease resistance (10,39).

Development of genetic variation within a population must precede any selections of superior genotypes for crop improvement. Generation of genetic diversity within a breeding population is the most important limiting factor in plant breeding. Genetic diversity has been traditionally generated through the sexual cycle and mutagenesis (70).

Generation of genetic diversity through the sexual cycle is primarily accomplished by intra- and interspecies crosses. The incorporation of genes from one species into a second by hybridization and backcrossing (introgression) (40,85) may result in the incorporation of desirable traits without major alteration of a cultivated genotype. Spontaneous or induced polyploidy provides material in which multiple gene copies may demonstrate a gene dosage effect or exhibit simple dominance.

Recovery of variants through sexual recombination and mutagenesis is limited by several common problems. Mutations may remain undetected if they are recessive traits masked by dominant alleles. Mutations may reduce viability; therefore, mutant genotypes may persist at reduced frequencies. Structural differences in genotype may affect recombination (eg. translocations or inversions). Linkage may occur between desirable traits and undesirable traits (40) (eg. disease resistance and reduction in yield).

Hybridization is limited by the sexual compatibility of the species involved in the cross. Segregation products of interspecific hybrids are frequently inferior to parental types (40). Hybrids are often sterile (40). Sterility may be due to genotypically defective gametes caused by segregation difficulties in parents of different chromosome numbers and constitution by unbalanced hybrid genotype in zygotes, or by endosperm-embryo incompatibility (85).

Polyploid plants are frequently reduced in fertility (40). Consequently, they are of little value as breeding stock. Induction of chromosome doubling may restore fertility in amphidiploid plants (40).

In addition to restrictions imposed on a plant breeder by the variability available or generated within a breeding population, there are limitations imposed by time and space required to screen the material generated. Sexual recombination generates an enormous number of genotypes and phenotypes in segregating generations, even if parental genomes are similar (40). The plant breeder must screen this material and maintain accurate records to determine the genotypes of interest. Once material is selected, numerous generations are required for stabilization of the desirable traits. Field trials are necessary to determine

environmentally-induced variability. Seed must be propagated for maintenance of the line and distribution to growers. Finally, the breeder must satisfy the industrial and consumer specifications or new varieties will not be used.

Applications of cell culture techniques to plant breeding

Recent advances in the manipulation of cell cultures have led to guarded optimism for the selection and recovery of agronomically useful plant material from cell cultures. Variants recovered from cell culture may supplement the materials already available to the plant breeder. The establishment of in vitro axenic cultures of plant organs, embryos, cells, and protoplasts on chemically defined media have permitted the adaptation of microbial genetic manipulations to plant cell cultures. These techniques are predicated on the rapid expression of mutant characteristics at the cellular level. Screening of haploid cell populations has permitted the detection of recessive, nonlethal mutations (1).

Recovery of variants from cell cultures without selection pressure

Recovery of variants from cell cultures without exposure to selection pressure has been reported by several workers. This phenomenon is poorly understood and sometimes unrepeatable. The initial observation was the recovery of morphologically distinct plants from cell cultures of sugarcane (16,17). Recovery of plants that differ from parental materials in responses to pathogens and phytotoxins is of particular interest. Such variants have been recovered from cell cultures of sugarcane (15,16,17), potato (25,26), and maize (18). Sugarcane and potato are highly heterozygous crops that are clonally propagated. Maize is a diploid crop that is seed propagated. Variant traits are reported

-
I
C
t
C
P
a
C
i
P
(
Re

to be stable through vegetative propagation (26). Sexual segregation has not been demonstrated, precluding genetic analysis of the variance. A brief review of the literature follows.

Sugarcane (Saccharum officinarum) is a highly heterozygous, often mixoploid crop. Several workers have reported recovery of disease-resistant subclones from susceptible clones after passage in culture. Subclones resistant to Sclerospora sacchari (cause of downy mildew) and Physalospora tucumanensis (cause of red rot) (113) have been reported (16,17). Fiji disease-resistant subclones derived from Fiji disease-susceptible cv. Pindar were reported (15). One of the Fiji disease-resistant subclones was resistant to downy mildew (17). Disease-resistant variants also exhibited variations in germination rates and yield compared to the parental clone (17).

Commercial potato cultivars are members of the tetraploid subspecies Solanum tuberosum ssp. tuberosum (26). Vegetative vigor is dependent on heterozygosity. Yield, tuber morphology, tuber quality, tuber storage characteristics, foliage habit, environmental requirements for tuberization, and disease resistance are important agronomic traits of acceptable cultivars (26). Shepard (26) has reported recovery of variant potato protoclones from protoplast cultures of cv. Russett Burbank without the application of selection pressures. Twenty out of eight hundred protoclones were resistant to foliar inoculation with race 0 of Phytophthora infestans, a race that infects the parental cultivar. Some of these protoclones were also resistant to infection by a race of the fungus (race 1,2,3,4) that overcomes resistance conferred by several genes. Resistance was transmitted to vegetative progeny of the protoclones.

Matern, et al. (25) reported recovery of protoclones and their

Ca
Ce
Va
Si
C
3
t
S
f
d
-
-
-

vegetative progeny which exhibited a range of sensitivity to foliar treatment with a crude filtrate extracted from cultures of Alternaria solani. Response to filtrates correlated qualitatively, but not quantitatively with the number of lesions. Shepard (26) reported that attempts to repeat the purification of the A. solani toxic complex were unsuccessful. Of the original A. solani filtrate-treated protoclonal lines, five clones out of 500 were significantly more resistant to the filtrate than the parent clone; one out of 500 was more sensitive than the parent clone. Four of the five 'toxin'-resistant protoclonal lines had enhanced resistance to A. solani (25,26).

The adventitious recovery of toxin-resistant plants from Texas male-sterile (TMS) cytoplasm maize callus without selection pressure has been reported by Brettel, et al. (18). Host-specific toxin from Drechslera maydis (Nisikado) Subram. & Jain (= Helminthosporium maydis Nisikado) race T was used in serial subculture selections of TMS-cytoplasm corn in an attempt to confirm the work of Gengenbach, et al. (2,34). Brettel included nonselected TMS-cytoplasm controls. Fifty-eight toxin-resistant male-fertile plants were recovered from the selected callus (18). Thirty-one similar plants were recovered from the nonselected TMS-cytoplasm control callus (18). The fact that almost 33% of the toxin-resistant male-fertile plants were recovered from culture without the application of specific selection pressure raises serious questions about the mechanisms responsible for generation of variants in culture and the role of biochemicals as selective agents in cell cultures. Toxin-resistance and male fertility did not appear to be caused by selection of N-cytoplasm-type mitochondria (18,37).

Numerous mechanisms may be postulated to explain the recovery of

f
n
n
t
E
e.
co
cy

variants from cell cultures without application of specific selection pressure. A popular explanation has been that the explant donor is chimeric or mixoploid tissue, and recovery of variants is due to isolation of subclones containing different alleles or different ploidy (15). Several mechanisms that contribute to the existence of genetic mosaics might explain the recovery of subclones of different ploidy.

a) Asynchronous mitotic division of two or more chromosome fractions might result in cells with different chromosome numbers. This might particularly explain the occurrence of cells with very low chromosome numbers (15). b) The coexistence of several distinct chromosome populations within a particular tissue may be postulated (15,90).

Environmental selection pressure (i.e. cell culture conditions) would determine the frequency of each subpopulation (15). c) Supernumerary chromosomes might be irregularly distributed during mitosis (15).

Although these mechanisms may explain some of the variability recovered from mixoploid tissue, they may not be applicable to variants recovered from nonmixoploid tissue. Variants have been recovered from nonmixoploid tissues of sugarcane (91), maize (18), and potato (25,26).

Spontaneous endopolyploidy has been observed in leaf mesophyll tissue from the onset of differentiation, while root and shoot meristems remained consistently diploid (88). Spontaneous endopolyploidy was reported to increase from the early stages of leaf primordia through the fully differentiated stage of maximum cell enlargement (87).

Endopolyploidy has been induced by decapitation (87), NaCl (87), and exposure to short day photoperiods (87,88). Endopolyploidy appears correlated with increase in succulence (87) and accumulation of cytokinin-type growth regulators (87). It seems reasonable to postulate

the spontaneous and induced occurrence of endopolyploidy in cell cultures. Decapitation or excision to produce explants, incorporation of inorganic salts and plant hormones in the culture medium, and choice of light regimen may parallel or enhance induction phenomena reported in intact tissues. Numerous workers have observed spontaneous variation in chromosome numbers after passage through culture. Plants recovered from culture which express a novel character must be examined cytologically to verify this hypothesis.

Another popular explanation proposed for generation of variants in cell culture is the occurrence of chromosomal mutations (15,26). Cytological examinations are necessary to provide correlative data for this explanation. Cytological examination of variants occurring in cell cultures has been reported by only one worker (8,15).

Recombination in the mitochondrial genome has recently been claimed (92). This warrants consideration as a mechanism in generation of variants for extranuclear encoded traits such as sensitivity to Helminthosporium maydis race T and Phyllosticta maydis toxins (18,36).

Low molecular weight episomal mtDNAs have been reported in S (101), T, C, and N (37) cytoplasms of Zea mays (maize). Reversion of S-cytoplasm maize to male fertility is accompanied by loss of these episomal mtDNAs (110). It is possible that the episomal mtDNAs are incorporated into the high molecular weight mtDNA (36). It seems reasonable to postulate similar mechanisms for other cytoplasmically-encoded traits. Examination of the frequency of episomal mtDNAs, and determination of their fates in other species might indicate whether or not this is a significant mechanism for generation of variants in cultures.

The possibility of automutagenesis has been suggested by Shepard (26), based on the observation that protoplasts have a minimum density plating requirement. Shepard (26) has suggested that dead protoplasts release potentially mutagenic flavonoids when plating density is below the threshold level. The implication is that surviving protoplasts are mutagenized and eventually recovered as variants. This should be tested with a rapid and quantitative system such as the Ames test (115).

Finally, epigenetic variation should be considered as a possible factor in differential gene expression and recovery of variants (26). In an examination of the phenomenon of cytokinin habituation of tobacco cell cultures, Meins (89) proposed that habituation involved the stable expression of normally unexpressed genetic potential. This was based on observations that cytokinin habituation occurred at rates 100 to 1000 times faster than somatic mutation rates in tobacco (98), was readily reversible, and did not eliminate the totipotency of altered cells (89). Meins presented indirect evidence for the maintenance of the habituated state by a positive feedback loop involving an autocatalytic regulatory molecule (89). Autocatalytic synthesis of regulatory molecules in plant cells has been reported for ethylene (102), cyclic-AMP (103), and indole acetic acid (104).

The expression of disease and herbicide resistance in cell cultures has been shown to be altered by the culture medium (22,23,99). Susceptible phenotypes have been induced in resistant genotypes. This work demonstrated that environmental factors can activate or perhaps suppress expression of genes. Thus, we should consider the possibility that variability expressed in and recovered from cell cultures may result from epigenetic phenomena induced by unknown autocatalytic regulatory

molecules. Certainly the concentration of culture medium constituents might be sufficient to induce autocatalysis of a regulatory molecule in a positive feedback loop. To determine the role of epigenetic phenomena in the generation of variants in culture, plants must be regenerated from the variant cultures. The regenerated plants must undergo sexual segregation and there must be a genetic analysis of subsequent progeny.

Traits expressed in intact tissue and *in vitro*

There are several reports of positive correlations between traits expressed in intact tissue and *in vitro*. These traits are generally under single gene control and are not tissue specific.

Ingram (31) reported that genotype differential sensitivity to *Phytophthora infestans* (Mont.) DeBary race 4 is expressed in callus cultures of *Solanum tuberosum* resistant cv. Orion (R_1) and susceptible cv. Majestic (rr). Callus from cv. Orion was colonized by *P. infestans* race 1, but restricted fungal growth of race 4. Callus of cv. Majestic was aggressively colonized by both races of fungus. This was comparable to intact responses to both races of the pathogen. Growth of race 4 germ tubes was inhibited by suspension cultures of cv. Orion, but not by cv. Majestic. Suspension culture filtrate of cv. Orion, assayed 24 hours or more after inoculation with race 4 sporangia, was toxic to germinating zoospores of *P. infestans* races 1 and 4, but harmless to spores of *Botrytis allii* or *Glomerella cingulata*. Similar culture filtrates obtained from inoculated suspensions of cv. Majestic had no inhibitory effect on zoospore germination. Evidence for expression of R_1 resistance in culture is provided by genotype differential race-specific response to the fungal pathogens.

Warren and Routley (19) reported partial expression of the Ph₁ gene for resistance to Phytophthora infestans race 0 in tomato callus. Callus derived from tomato cultivars Rockingham and New Hampshire Surecrop, homozygous for Ph₁, supported fungal growth without color or textural changes in the callus. No differences in the extractable phenolic substances were detected between inoculated and control callus. Callus derived from cvs. New Hampshire Victor and Johnny Jumpup, which lack the Ph gene, succumbed to infection and had almost complete loss of extractable phenols by ten days after fungal infection. Chromatographic determination of phenols prior to and including six days after inoculation did not indicate significant differences between genotypes or treatments. The data are insufficient for determination of the role of phenols in fungal resistance.

Helgeson, et al. (20,21) reported that a single dominant gene for resistance to Phytophthora parasitica Dast. var. nicotianae (Brede de Haan) Tucker race 0 was expressed in callus culture. Near isogenic lines of Nicotiana tabacum exhibited differential rates of fungal colonization in pith callus cultures and rooted cuttings obtained from homozygous parental, F₁, F₂, and F₃ generations. All callus and rooted cuttings were susceptible to infection by race 1 of the pathogen. Haberlandt, et al. (22) reported that phenotype was affected by callus morphology and age, incubation temperature after zoospore inoculation, inoculum concentration, and auxin-cytokinin ratio in the culture medium. Alteration of any of these factors resulted in expression of a susceptible phenotype by a resistant genotype.

Selections for biochemical resistance in cell cultures

The chance of recovering a particular variant trait may be increased by screening a population for resistance to a selective agent or condition. The general procedures for biochemical selection of variants, and determination that the selected trait is not an epigenetic phenomenon, are outlined here. Putative resistant mutants are generally selected from a cell population in one of two ways: a) haploid cells or protoplasts are subjected to a lethal dose of the selective agent and survivors are identified; or b) cells with normal ploidy are exposed to stepwise increments of a selective agent in a sublethal enrichment procedure. Cell populations are sometimes exposed to mutagenic agents prior to selection to enhance the mutation rate. Selected variants are cultured in the absence of selective pressure, then reintroduced to selective media to demonstrate stability of the variant trait. Plants are regenerated from the variant cell lines and are examined for expression of the selected trait in vivo. Transmission of the selected trait through sexual segregation and determination of the pattern of inheritance are the final steps. Expression of the totipotency of the selected cell lines is often a limiting factor in distinguishing genetic from epigenetic phenomena.

Selections for resistance to nonspecific toxins in cell cultures

Several fungal and bacterial pathogens produce nonselective toxins that are correlated with virulence. Several nonspecific toxins have been identified as disease determinants and consequently are valuable as potential selective agents for cell culture screening of toxin and disease-tolerant cell lines. In addition, selections have been

successfully performed using a toxin-mimetic drug (1). Selections in culture using cell-free fungal culture filtrate of Phytophthora infestans have been reported (23,27), but will not be reviewed here because the data are inconclusive.

Pseudomonas tabaci is the causal agent of wildfire disease of tobacco. Conspicuous foliar symptoms are chlorotic halos surrounding necrotic lesions (53,86). The halo is caused by diffusion of tabtoxin into live cells and destruction of the chlorophyll (86). Cell-free culture filtrates reproduce the halo symptom. Tabtoxin has been isolated and characterized (38). Mutation analysis of the host indicates that the toxin is a significant factor in disease development (1,50). Pseudomonas angulata, causal agent of blackfire disease of tobacco, is considered to be a non-toxin producing variant of P. tabaci (86). Foliar symptoms of blackfire disease are distinguished from wildfire disease by the absence of chlorotic halos surrounding the necrotic lesions (86).

Carlson (1) selected mutagenized haploid cells and protoplasts of Nicotiana tabacum cv. Havana Wisconsin 38 that were resistant to 10 mM methionine sulfoximine (MSO) in the culture medium. MSO elicited chlorotic halos on tobacco leaves similar to characteristic symptoms of P. tabaci infection. MSO resistance was retained after passage on nonselective medium. Diploid plants were regenerated from the resistant callus, treated with MSO, and inoculated with P. tabaci. Inoculated leaves exhibited necrotic lesions, but lacked chlorotic halos. Bacterial multiplication and toxin production were not inhibited in MSO-resistant plants. MSO-resistant plants were susceptible to P. angulata infection. This suggested that selection for MSO-resistance concurrently selected for tabtoxin resistance. Preliminary inheritance studies indicated that

MSO-resistance was transmitted to F₂ progeny of the regenerated plants. This was the first report to demonstrate that selection for resistance to a toxin-mimetic drug permitted recovery of higher plant mutants tolerant to the toxin (1).

Pseudomonas phaseolicola is the causal agent of halo blight disease of beans. Symptoms include systemic chlorosis or discrete lesions surrounded by chlorotic halos. Races 1 and 2 of the pathogen produce phaseolotoxin, but differ in host range (50). Mutant strains of P. phaseolicola which do not produce phaseolotoxin grow normally in leaves, but differ from wild type isolates by the absence of evident chlorotic halos and systemic infection (50). This suggests that phaseolotoxin is a factor that contributes to virulence (50).

Bajaj, et al. (24) monitored growth of callus tissues of Phaseolus vulgaris cv. Manitou on medium containing culture filtrates of P. phaseolicola, P. syringae, or P. morsprunorum, a nonpathogen of beans. Callus growth was significantly reduced on media containing culture filtrates of all three bacteria. Callus grown on medium amended with P. phaseolicola culture filtrate had higher levels of ornithine than control callus. The alteration in ornithine level paralleled reported increases in P. phaseolicola-infected leaf tissue (116). This suggests that this system is feasible for selection of disease tolerant variants.

Phoma tracheiphila (Petri) Kantschaveli & Gikachvili is the causal organism of mal secco disease of citrus. An extracellular glycoprotein isolated from culture filtrates has been identified as a toxin with the capability to induce disease symptoms in Citrus jambhiri (54). Avocado, cucumber, and tomato are also sensitive to the toxin (54). Nachmias, et al. (19) incorporated the purified glycoprotein into culture medium.

Callus was initiated from ovaries of Citrus lemon cv. Eureka and Citrus sinensis cv. Shamouti and scored for toxin-induced growth inhibition. Lemon callus showed significant inhibition at toxin concentrations that did not affect orange callus. This correlated with observations of susceptibility and resistance to mal secco disease. Toxin concentrations inhibitory to lemon callus appear high (0.3 to 0.6 mg/ml), but orange callus tolerated 2.4 mg/ml without inhibition. Attempts are reportedly underway to use this toxin as a selective agent for recovery of mal secco-resistant plants from cultures (B. Nadel, private communication).

Selections for resistance to host-specific toxins in cell cultures

Fourteen species of pathogenic fungi have been reported to produce low molecular weight compounds that are selectively toxic to certain genomes of the host plant (49,50,51). These host-specific toxins (51) are implicated as determinants of pathogenicity and virulence (50). Physiological, histological, biochemical, and genetic data have been examined in attempts to evaluate the role of these toxins in disease (49,50,51). Criteria for evaluation of these toxins in disease etiology include: correlation of virulence with toxin production, comparable specificity of toxin and pathogen, toxin-induced symptoms in host plants, isolation of toxin from diseased plant material, genetic analysis of the pathogen and its host (50). Host-specific toxins may be accepted as microorganism surrogates, if one defines disease in biochemical terms as a metabolic disruption resulting from chemical lesions (51). The host-specific toxins can be used as biochemical pathogen surrogates to screen for disease resistance in vivo and in vitro (39,50).

Helminthosporium maydis (Nisikado & Miyake) race T is the causal

agent of southern leaf blight of corn. Susceptibility to the fungus is conditioned by nuclear and cytoplasmic genes (28). Maize with Texas male sterile (TMS) cytoplasm is more susceptible to the fungus than is maize with nonsterile (N) cytoplasm (28). Crosses between pathogenic race 0 and pathogenic, toxin-producing race T yielded progeny with parental ditypes (111). Race 0 has a low level of virulence on both TMS and N-cytoplasm maize and does not produce a toxin. Race T has a high level of virulence on TMS-cytoplasm maize, but a low level of virulence on N-cytoplasm maize. Progeny segregation for virulence indicated that T-toxin may not be required for pathogenicity, but clearly is a virulence factor (111). Sensitivity to T-toxin is cytoplasmically determined (28). Cytoplasmic factors conditioning toxin sensitivity condition increased susceptibility to race T of the pathogen (28). Nuclear genes condition fungal susceptibility, but are reported to have no effect on toxin-sensitivity (28). Compatibility between race T and susceptible maize genotypes appears to be independent of sensitivity to T-toxin, although toxin-sensitive cytoplasm exhibits increased levels of the disease (28). T-toxin must be considered a host-specific toxin with respect to cytoplasmic determinants, but not with respect to nuclear genomes (28). Additionally, the relative sensitivities of genotypes to T-toxin is reported to be influenced by the choice of bioassay (28,112). These factors suggest that use of the toxin for disease-resistance screening must be done cautiously.

T-toxin has been used as a selective agent for the recovery of toxin-resistant maize callus (2,18,34,35,36). Mitochondria isolated from control N-cytoplasm callus and toxin-resistant TMS-cytoplasm callus were insensitive to T-toxin as determined by swelling, NADH oxidation,

oxidative phosphorylation (34), and malate-driven 2,6-dichlorophenol indophenol reduction (34,35). Control TMS-cytoplasm mitochondria were sensitive to toxin effects on all four parameters (34). T-toxin resistance was stable in the absence of selection pressure (34,35). All plants regenerated from T-toxin-resistant TMS-cytoplasm callus were resistant to T-toxin and to H. maydis race T (2,18,35,36). T-toxin-resistant regenerated plants were also resistant to Phyllosticta maydis toxin (36). Toxin-resistance was maternally transmitted through three generations of regenerated plants (36).

Helminthosporium sacchari is the causal agent of eyespot disease of Saccharum officinarum (sugarcane). Although the structure of the fungal toxin and its mode of action have been the object of intensive investigation (105-108), there are still questions about its relationship to pathogenicity (50). Host-specificity is the primary criterion cited for pathogenicity (50). Irregularities in clonal response to pathogen and toxin have been reported (109,114). Nevertheless, suspension cultures of a susceptible clone, with and without prior mutagenesis, were treated with crude preparations of H. sacchari toxin in attempts to select toxin-resistant sugarcane clones. Plantlets were regenerated from the suspension cultures and screened at maturity for field resistance to the toxin. The results have been equivocal (17). The physiological effects of the toxin on suspension cultures were not reported.

Host-specific toxin-producing pathotypes of Alternaria alternata

Of the 14 known fungi that produce host-specific toxins, two of those belonging to the genus Alternaria have been the objects of intensive investigation. Although mutation analyses of the pathogens have

not been reported, there is considerable correlative evidence for toxin involvement in pathogenicity and virulence. Several of the toxins have been isolated and examined for biological activity, but no workers have reported use of these toxins in cell culture for disease screening or physiological studies. This section is a brief introduction to several of the host-specific toxin-producing Alternaria spp., including A. alternata f. sp. lycopersici, the toxin-producing fungus of this study.

Non-specialized forms of Alternaria alternata (= A. tenuis Nees) are saprophytes or weak pathogens on numerous hosts. They cause disease in mature and senescent tissue (57). The fungus may exist as a symptomless latent infection in live plant tissue (57). Specialized, highly virulent forms of the fungus are known. Germinating spores of virulent and avirulent isolates are capable of penetrating mature or senescent tissue, cellophane, and collodion membranes (57). This suggests that pathogenicity is independent of aggressive penetration of plant tissue (57). Specific pathogenicity has been attributed to host-specific toxin production (73).

Tenuazonic acid, a phytotoxic metabolite of Alternaria spp. (58), is produced by avirulent isolates and is not released during germination of virulent spores (73). It is not considered to be a primary determinant of disease (73). Attempts to correlate responses of intact tobacco leaf tissue with suspension culture responses to tenuazonic acid have been unsuccessful (T. Kumashiro, private communication).

Highly pathogenic Alternaria spp. have been classified according to host-specificity, but are morphologically similar enough to be reclassified as pathotypes of A. alternata (50,56,57,73). Fungal pathogenicity and host-specific toxin production are interdependent. Fungus that is

attenuated in toxin production is nonpathogenic, but retains the ability to penetrate tissue surfaces (57). Attenuated fungus has been reported to exist in a saprophytic state (50). Saprophytes in culture have been reported to mutate to pathogenicity with accompanying resumption of toxin production (50). A. kikuchiana (57,62,76), A. mali (61,74,79), A. citri (55), A. alternata f. sp. lycopersici (56,69,71), A. fragariae (50), and A. longipes (50) have been identified as host-specific toxin-producing members of A. alternata. Toxin characteristics of four of these pathotypes will be summarized.

A. mali Roberts is the causal organism of Alternaria blotch of apple, an economically serious disease of apples in Japan (60), first recorded in 1956. It is particularly deleterious to certain cultivars of Malus pumila Miller var. domestica Schneider (60). Susceptibility to the fungus is controlled by multiple dominant genes (74).

Two major and five minor host-specific toxins have been identified from different pathogenic isolates (60). Not all minor toxins are produced by all pathogenic isolates. Toxicity of minor toxins is less than that of major toxins (60). Major toxins have been chemically characterized as depsipeptides. AM I-toxin (alternariolide) is cyclo- α -hydroxy-isovaleryl- α -amino-p-methoxyphenylvaleryl- α -amino-acryl-analyl-lactone (79); AM-II toxin is a demethoxy derivative of AM I-toxin (79).

The host range of A. mali may vary with isolate-specific production of major and minor host-specific toxins. This implies that the loss of ability to produce one host-specific toxin does not necessitate loss of pathogenicity. Nijisseiki pear is susceptible to A. mali (59).

A. mali toxins were produced by germinating spores of virulent isolates (60). Germinating virulent spores induced electrolyte leakage

only in susceptible tissues (60). Toxin-induced leakage was concentration-dependent (60). Resistant tissues exhibited similar loss of electrolytes at toxin concentrations 1000-fold greater than those required for response of susceptible tissue (60). AM I-toxin induced plasma membrane modification and transformation of chloroplast grana lamellae to vesicles in mesophyll and bundle sheath cells of susceptible apple tissue (67). Toxin treatment appeared to have no effect on the ultrastructure of plastids in epidermal or phloem cells (67).

Toxin-insensitivity was induced by heat pretreatment of susceptible and resistant tissues. Heat pretreatment provided protection against necrosis and electrolyte leakage (61).

Alternaria kikuchiana Tanaka was identified as the causal agent of black spot disease of Japanese pear (Pyrus serotina Rehd.) in 1933. Susceptibility to A. kikuchiana is dominant and is under single gene control (76). Analysis of 22 susceptible cultivars indicated that all were heterozygous for this locus (76). Homozygous susceptible cultivars may be seedling lethals (76). Sensitivity to A. kikuchiana host-specific toxins (AK-toxins) is controlled by the same locus that conditions fungal susceptibility (77). Resistant cultivars and nonhost tissue are essentially immune to the fungus and its toxins (77). Susceptibility is derived from the cv. Nijisseiki (57,76).

Virulent isolates are pathogenic only to immature tissue of susceptible cultivars (57). Virulent isolates produce at least three host-specific toxins which have identical host-specificity as the fungus (57,73). Loss of pathogenicity is accompanied by loss of AK-toxin production (57). Mutation to pathogenicity is accompanied by resumption of toxin production (50).

AK-toxins are implicated in successful establishment of infection in a similar manner as Helminthosporium victoriae (93) and Helminthosporium carbonum (80) toxins. Spore germination, mycelial growth, and formation of appressoria are similar on resistant and susceptible tissue (62). Germinating virulent spores produce toxins which induce necrosis and electrolyte leakage in susceptible tissue (62).

AK-toxins have been examined in detail for physiological effects on host tissue. AK-toxins are reported to affect plasma membrane function of susceptible cultivars, as evidenced by rapid loss of electrolytes (62,64,65). AK-toxins are also reported to inhibit germination of pollen grains from susceptible cultivars (77), induce cessation of cyclosis (77), induce stomatal closure (77), and induce rapid loss of plasmolytic ability in isolated susceptible cells (77).

Reduction of toxin sensitivity in susceptible tissue was accomplished by pretreatment with EDTA (66), heat (63), N₂ atmosphere (73), iodoacetamide (-SH binding agent) (73), and N,N-dicyclohexylcarbodiimide (energy transfer inhibitor) (73).

Alternaria citri Ellis & Pierce pathotypes are specific to Citrus reticulata (Dancy tangerine) and Citrus jambhiri (rough lemon) (55). The isolates produce host-specific toxins that are specific only to the host plants (55).

Two host-specific toxins isolated from pathotypes virulent on rough lemon induced water soaking, necrotic lesions, and veinal necrosis (55). Nonhost species, including Dancy tangerine, tolerated 10,000-fold greater concentrations of toxin than did the susceptible rough lemon cultivars (55). One host-specific toxin has been isolated from the pathotype virulent on Dancy tangerine. Both toxins induced electrolyte leakage

from their respective hosts, with no measurable effects on nonhost tissues (55).

A. alternata f. sp. lycopersici was identified as the causal agent of a stem canker disease of tomatoes (Lycopersicon esculentum Mill.) in California in 1975 (68). The disease was reported in Japan in 1980 (71), and the same pathotype was identified as the causal agent (71). Disease symptoms include dark stem cankers with concentric zonation that enlarge slowly to girdle the stem, brown dry rot in pith subtending cankers, discoloration of xylem in the cankers, discoloration of pith adjacent to primary xylem flanking cankers, petiole epinasty, inward rolling of leaflets, and unilateral or bilateral angular necrosis of leaflets (68). Disease resistance is controlled by a single dominant gene (68). Susceptibility in California tomato varieties is derived from the cv. Pearson (68). Seed and seedlings in the cotyledonary stage were refractory to inoculation with spore suspensions (68). Plants in the second true leaf stage and older were susceptible to infection (68).

At least two forms of the host-specific toxin have been isolated and characterized (69,70,71) from cell-free culture filtrates of pathogenic isolates. The proposed structures of the toxins are 1,2,3-propanetricarboxylic acid esterified to either 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol or 1-amino-7,15-dimethylheptadeca-2,4,5,13,14-pentol (69,70). The toxins exhibit the same host-specificity as the pathogen (56,69,70).

Kohmoto (71) has compared host-specific toxins from American and Japanese pathotypes for host range, symptoms, and chromatographic behavior. Biological activity of the isolates is identical (71). The multiple host-specific toxins are similar chromatographically (71) and

structurally (K. Kohmoto, private communication).

The host-specific toxins elicit foliar symptoms characteristic of natural infection (56). Resistant tomato tissue tolerates at least 1000-fold higher concentrations of toxin than susceptible tissue (56). Ultrastructural examinations of toxin-treated leaf tissue revealed that toxin affects mitochondria and rough endoplasmic reticula only in susceptible genotypes (67). Dosage-dependent electrolyte leakage is detectable only after the onset of cellular necrosis (72). There is a claim that the toxin acts as a competitive inhibitor of aspartate transcarbamoylase (McFarland, private communication). Genotype differential sensitivity of aspartate transcarbamoylase to toxin was reported (72). Amino acids of the L-aspartate family and intermediates of aspartate metabolism gave protection against the toxin in vivo (72).

Genetic determination of fungal resistance is reportedly controlled by a single dominant gene (56). Determination of toxin sensitivity is reportedly controlled by a single locus with two alleles expressing incomplete dominance (56). Genetic control of pathogen and toxin sensitivity are thought to be controlled by the same locus (70). Published data for this conclusion are insufficient and further examination is required.

Limitations of cell culture studies

In vitro and intact tissue responses to selective agents must be correlated to demonstrate that plants regenerated from cell cultures (subjected to biochemical selections pressure) have heritable, stable traits. Plant cell cultures are relatively homogeneous, cuticle-free, metabolically active cell populations. The characteristics which make

cell cultures advantageous may also be the source of limitations. Keen and Horsch (33) examined production of the antifungal pterocarpan 6a-hydroxyphaseollin in near-isogenic lines of Glycine max (L.) Merr. The resistant cv. Harosoy 63 and the susceptible cv. Harosoy were inoculated with Phytophthora megasperma Drechs. var. sojae Hildb. Hypocotyls, cotyledons, roots, and callus initiated from seedling hypocotyls were inoculated with zoospores. Cultivar-specific differential accumulation of 6a-hydroxyphaseollin occurred only in hypocotyls. Ingram (32,39) reported variable responses in and poor correlations between intact tissue, callus, and root cultures of resistant and susceptible Brassica spp. inoculated with Peronospora parasitica (Fr.) Tul. Similar variable results were reported for Beta vulgaris inoculated with Peronospora farinosa (Fr.) Fr. f. sp. betae (39).

Growth phases of cell cultures have been shown to differ metabolically and in physiological responses to selective agents. Sugarcane suspension cultures in log phase were more sensitive to high temperature than were stationary phase cultures (8). Log phase cultures had a higher respiration rate than did stationary phase cultures (8). Dikegulac (a growth regulator) was toxic to log phase cultures of Compositae species, but had no effects on stationary phase cultures (3).

Cell cultures are cuticle-free. Compounds that affect penetration or transport may elicit different responses in cell cultures than in intact plants (42). Cell cultures may be chlorophyllous or achlorophyllous. Compounds affecting photosynthesis should be examined in both types of culture for correspondence with intact plant tissue (41,42).

The occurrence of different secondary metabolic pathways or regulatory enzyme sensitivities must be considered. Metabolism of cisanilide

(an herbicide) is different in suspension cultures and in excised leaves of carrot and cotton (94). Widholm (48) has reported differences in enzyme sensitivity to feedback inhibition in plants and in cultures derived from these plants. Aspartokinase sensitivity to lysine-threonine inhibition differed in roots and root-derived suspension cultures of Daucus carota, and cotyledons and suspension cultures of Glycine max. Homoserine dehydrogenase sensitivity to threonine inhibition differed in soybean callus and cotyledons. Cell lines of Nicotiana tabacum resistant to 5-methyltryptophan exhibited altered anthranilate synthetase sensitivity to inhibition by tryptophan and 5-methyltryptophan. Plants regenerated from the resistant cell lines did not exhibit altered anthranilate synthetase sensitivity; callus and suspension cultures derived from the regenerated plants did exhibit altered enzyme sensitivity. This trait was selected and expressed in culture, but was transmitted through regenerated intact plants without expression.

The last major consideration is medium-induced alteration of phenotype. Haberlach, et al. (22) have reported induction of a susceptible phenotype in Nicotiana tabacum callus cultures derived from a genotype resistant to Phytophthora parasitica race 0 by alteration of the auxin/cytokinin ratio in the culture medium. Oswald (95) has reported induction of a susceptible phenotype in a metribuzin-resistant genotype of soybean by alteration of the sucrose and inositol concentrations in the culture medium. Alteration of phenotypic expression of susceptible genotypes has not been reported.

MATERIALS AND METHODS

Tissue culture media

All media were modifications of Linsmaier and Skoog medium (81) prepared with double distilled, deionized water and modified to contain, per liter, 100 mg myo-inositol; 30 g sucrose; vitamins; and hormones. Bacto-agar (Difco, 0.9%) was added to specific liquid media to prepare solid media for plating. All media were steam sterilized at 121°C. Solid media were poured into 100 x 15 mm plastic Petri plates and sealed with parafilm after transfer of tissue. Liquid media aliquots, 50 ml per 125 ml Ehrlenmeyer flask, were stoppered with foam plugs.

The basic medium was modified for seed germination to contain, per liter: 1 mg thiamine hydrochloride; 0.5 mg pyridoxine hydrochloride; 0.5 mg nicotinic acid; 9 g Bacto-agar; pH 6.0.

The basic medium was modified for initiation and maintenance of cell cultures (culture medium R2A) to contain, per liter; 1 mg thiamine hydrochloride; 0.5 mg pyridoxine hydrochloride; 0.5 mg nicotinic acid; 2 mg indole-3-acetic acid; 2 mg 2,4-dichlorophenoxyacetic acid; 0.3 mg kinetin; pH 6.0.

The basic medium was modified for initiation and maintenance of cell cultures (culture medium T-12) to contain, per liter: Nitsch vitamins (82); 3 mg indole-3-acetic acid; 2 mg 2,4-dichlorophenoxyacetic acid; 0.1 mg benzyladenine; pH 5.8.

The basic medium was modified for maintenance of suspension cultures (culture medium 3/.3) to contain, per liter: 1 mg thiamine hydrochloride; 3 mg indole-3-acetic acid; 0.3 mg kinetin; pH 6.0.

Additional media were screened to obtain proper conditions for initiation and support of callus growth. Twenty-seven "T-series" media were prepared with all possible combinations of hormone concentrations listed. "T" media were prepared from basic medium modified to contain, per liter: Nitsch vitamins (82), 10 mg, 3 mg, or 1 mg indole-3-acetic acid; 2 mg, 0.5 mg, or 0.1 mg 2,4-dichlorophenoxyacetic acid; 1 mg, 0.3 mg, or 0.1 mg benzyladenine, 9 g Bacto-agar; pH 5.8. Culture medium R3 was prepared from basic medium modified to contain, per liter: 1 mg thiamine hydrochloride; 0.5 mg pyridoxine hydrochloride; 0.5 mg nicotinic acid; 5 mg indole-3-acetic acid; 0.5 mg 2,4-dichlorophenoxyacetic acid; 0.3 mg kinetin; 9 g Bacto-agar; pH 6.0. Culture medium R3B was prepared from basic medium modified to contain, per liter: 1 mg thiamine hydrochloride; 0.5 mg pyridoxine hydrochloride; 0.5 mg nicotinic acid; 2 mg naphthalene- α -acetic acid; 1 mg benzyladenine; 9 g Bacto-agar; pH 6.0.

Cell culture initiation and maintenance

Seeds of Lycopersicon esculentum cvs. Earlypak-7, VFN-Bush, Walter, and Ace, were kindly provided by the Peto Seed Company, Saticoy, CA. Seeds were surface-sterilized by immersion in 1.31% sodium hypochlorite aqueous solution (Chlorox diluted 1:3) for 15 minutes, then rinsed three times with copious volumes of sterile distilled water. Seeds were plated on germination medium R5 and incubated in the dark at 27°C for seven days, or until the cotyledons were fully expanded. Cotyledons and 5 mm hypocotyl explants were plated on solid culture media and held under

cool-white fluorescent lights (150 - 280 foot-candles) at 22-24°C, with 16 hour photoperiods. Friable callus was subcultured every three to four weeks. Stock callus was also maintained by plating suspension cultures in late exponential or stationary phase and subculturing as described. Suspension cultures were initiated by submersion of friable, achlorophyllous callus (~1.0 cm³) in appropriate liquid media. Suspensions were initiated and maintained in the dark on gyrorotatory shakers, 125 rpm, 23-25°C. Established tomato cell suspensions were transferred by equal volume dilution with fresh liquid medium every third day, to maintain the cultures in exponential growth phase. Tobacco cell suspensions were transferred by dilution with three volumes of fresh media every third day, to maintain the culture in exponential growth phase.

Stem canker-resistant cv. Walter and stem canker-susceptible cv. Earlypak-7 were maintained on culture medium R2A. Stem canker-resistant cv. Ace and stem canker-susceptible cv. VFN-Bush were maintained on culture medium T-12. Tobacco suspension cultures (obtained from Dr. Russell Malmberg) were maintained on culture medium 3/.3.

Fungal cultures

Alternaria alternata f. sp. lycopersici isolates designated AS-27 and AS-27-3 gen. 17 were obtained from Dr. David Gilchrist, U.C., Davis. Cultures were maintained in the laboratory on potato dextrose agar at 22°C, or on corn meal agar at 27°C in the dark to obtain sporulating cultures for toxin production. Stock fungal cultures were maintained on potato dextrose agar or corn meal agar at 4°C.

Toxin preparation

Cultures were grown in 1000 ml Roux bottles each containing 200 ml of culture medium (per liter: 1.184 g glutamine; 0.100 g NaCl; 1.000 g K_2HPO_4 ; 0.500 g $MgSO_4 \cdot 7H_2O$; 0.130 g $CaCl_2$; 0.500 g Yeast extract (Difco); 900 ml double-distilled, deionized water; pH 6.0 steam sterilized at 121°C and supplemented with sterile 20.7 g glucose in 100 ml water (Gilchrist, private communication)). Medium was inoculated with 50-100 mm² plugs of sporulating fungus. Stationary cultures were maintained at 22°C for 15 to 17 days with a 12-24 hour photoperiod. Cultures were harvested by filtration through cheesecloth and Whatman no. 1 filter paper. Cell-free filtrates were concentrated to 1/20 original volume under reduced pressure. Dissolved solids were precipitated from the filtrate with two volumes of methanol held overnight at 4°C. The methanolic supernatant was filtered through Whatman no. 1 filter paper and the precipitate was discarded. Methanol was evaporated at reduced pressure and the residual aqueous volume was further reduced. The concentrated aqueous residue was partitioned three times against equal volumes of chloroform. The aqueous phase was then partitioned five times against equal volumes of water-saturated 1-butanol. The 1-butanol phase was evaporated to dryness under reduced pressure and the residue was dissolved in 75% methanol-25% water. Two ml aliquots of the methanol-soluble residue were serially applied to a 1.5 x 90 cm Sephadex LH-20 column equilibrated and eluted with 75% methanol. Flow rate was 0.22 ml per minute. The toxin was eluted between 92 and 132 ml. Fractions with toxin activity were identified by leaflet bioassay and thin layer chromatography with 60F-254 (Merck) fluorescing silica gel plates (0.25 mm thickness) developed with 1-butanol:acetic acid:water

(4:1:1). Three ninhydrin and bioassay positive spots were detected at R_f values of 0.35, 0.39, and 0.45. Toxin-containing fractions were pooled and evaporated to dryness under reduced pressure. The residue was dissolved in double-distilled, deionized water for use in tissue culture experiments. The toxin solution was sterilized by filtration through 0.20 μ Nalgene units. Toxin that was not required for immediate use was stored in 50% or 75% methanol at 4°C.

Toxin bioassay

A dilution end-point bioassay was developed to monitor toxin preparation. Seeds and seedlings in the cotyledonary stage were reported to be refractory to inoculation with Alternaria alternata f. sp. lycopersici spore suspensions (68); therefore, a semiquantitative detached leaflet bioassay was developed (61). Young, fully expanded leaflets were excised and placed on a moist substrate within a plastic-film wrapped enamel pan. Leaflets were wounded in the lamina and midrib with a fine gauge insect needle, then inoculated with a 10 μ l droplet of the preparation to be assayed. A tenfold dilution series was assayed on duplicate leaflets of resistant and susceptible tomato cultivars. The standard bioassay used leaflets from greenhouse grown resistant cv. Ace and susceptible cv. Earlypak-7. Assays were allowed to develop 4 to 6 days at ambient laboratory temperature and lighting. Leaflets were scored for veinal and intercostal necrosis. Differential toxin sensitivity was determined from the dilution end-points on susceptible and resistant cultivars. Resistant cv. Ace tolerated 1000-fold greater concentrations of toxin preparation than susceptible cv. Earlypak-7 at most steps in the toxin purification procedure.

Preparation of suspension cultures for toxin treatment

Suspension cultures of tomato cvs. Walter, Ace, and Earlypak-7 were routinely transferred (diluted with an equal volume of fresh medium) 24 hours prior to the initiation of an experiment. Suspensions of VFN-Bush and tobacco were transferred by dilution with three volumes of fresh medium 24 hours prior to initiation of an experiment. At the beginning of each experiment suspensions were filtered if necessary and three flasks of each cultivar were pooled to yield uniform cell inocula. Suspensions were pipetted to deliver two ml settled cell volume. Fresh medium was added to bring the final suspension volume to 50 ml. Suspensions were replaced on a gyrorotatory shaker for 43 hours. One ml of aqueous toxin stock solution, pH 6.0, was added to each suspension. Final toxin concentrations, by dry weight, in suspension cultures were between 200 ng/ml and 4 mg/ml. Double-distilled, deionized water was added as a control. Suspensions were maintained in the dark at 23-25°C on gyrorotatory shakers, 125 rpm. Most experiments were initiated with three replicates per treatment per determination of growth and mortality, and three times as many controls as toxin replicates per treatment. Most experiments were terminated after six days of exposure to toxin. All assays were destructive.

Growth determinations

Settled cell volume. Total volume of a suspension was transferred to a graduated centrifuge tube where cells were allowed to settle for thirty minutes. Results are expressed as the volume of settled cells per culture (98).

Dry weight. Cells which had been used in settled cell volume determinations were separated from the culture medium by filtration through

double thickness of Whatman no. 4 filter paper and dried for 48 hours at 100°C. Results are expressed as cell dry weight (mg) per culture.

Cell death

Samples of representative suspensions were removed at the initiation of a settled cell volume determination. Aliquots were stained (v:v) with 0.1% bromphenol blue (83), a mortality stain. The percentage of stained dead cells in a minimum population of 500 cells was determined by observation with an Olympus inverted microscope. On occasion, fluorescein diacetate (83) was used to confirm the viability of cells excluding the bromphenol blue stain. Cells were viewed under dark-field microscopy using a Zeiss exciter filter and barrier filters in various combinations (83).

Preparation of samples for spectroscopy and chromatography

Initial sample preparation consisted of a fivefold concentration under reduced pressure of the aqueous, cell-free supernatant of suspensions of cvs. Walter and Earlypak-7 that had been exposed to toxin for 120 hours. Subsequent determinations were performed on 15 ml aliquots of the same filtrates which were partitioned twice with equal volumes of ethyl acetate - water. The ethyl acetate phase was retained and evaporated to dryness under reduced pressure. The residue was dissolved in 0.5 ml double-distilled methanol. Ten μ l of the methanol soluble residue was diluted with 0.3 ml double-distilled methanol for spectroscopic examination. Samples for chromatography were used without further dilution.

Thin layer chromatography. Droplets (10 μ l) of the samples (prepared as described) were spotted on 60F-254 (Merck) fluorescing

silica gel plates (0.25) mm thick) and developed twice by thin layer chromatography with chloroform:methanol:water (65:25:4). Compounds were viewed with long and short wave UV-light.

Paper chromatography. Droplets (20 μ l) of the concentrated aqueous filtrates, or the methanol soluble residue of the concentrated aqueous filtrates, were spotted on Whatman no. 1 chromatography paper. Chromatograms were developed with 1-butanol:ethanol:water (4:1.5:2.2); acetic acid:water (15:85); or 1-propanol:ammonium hydroxide (7:3). Compounds were detected with long and short wave UV-light, Arno's reagent, and concentrated sulfuric acid. Standards (5 μ l) of chlorogenic acid, caffeic acid, ferulic acid, para-coumaric acid, and rishitin solutions were chromatographed with the samples to serve as markers.

Spectroscopy. Absorption spectra were measured with a single beam spectrophotometer consisting of a Cary 14R monochromator with a tungsten or deuterium light source, photomultiplier tube, and photometer on line with a Hewlett-Packard 2108mx minicomputer. This instrument has been described in greater detail by Manabe and Poff (1978) (84). Samples consisted of 0.3 ml of a methanolic solution of the unknowns in a stainless steel cuvette with a quartz window on the bottom. The measuring beam was directed vertically through the sample (optical path length 2.3 mm). Absorbance values were digitized at 0.2 nm or 0.4 nm intervals and deposited in the memory of the computer. Absorption spectra could be manipulated by the computer to calculate difference spectra correcting for the spectrophotometer system, the solvent, and in some cases, to correct for the control samples. The presented data were plotted directly by the computer using an x-y recorder.

RESULTS

Evaluation of toxin preparation

The specific activities of the toxin preparations used in the suspension culture experiments were calculated from dry weight and dilution end-point bioassay data for each preparation. Specific activity was defined as the dry weight of the preparation at the dilution end-point for the susceptible cultivar Earlypak-7. The toxin preparation used in the initial experiments was prepared from fungal isolate AS-27 and gave an assay end-point at 11 ng/ml. Toxin used in most of the experiments was prepared from fungal isolate AS-27-3 gen. 17. Specific activity of this preparation was calculated to be 12 ng/ml. Both toxin preparations contained three distinct fractions with toxin activity, as determined by leaflet bioassay and ninhydrin positive reaction on TLC plates (1-butanol:acetic acid:water, 4:1:1, v:v:v). Both toxin preparations were stable when stored in water or methanol at 4°C, within the detection limits of the bioassay and TLC.

Resistant cvs. Ace and Walter consistently differed in sensitivity to toxin, as shown by leaflet bioassays. Resistant cv. Walter tolerated at least 10-fold higher toxin concentrations (12 µg/ml) than did resistant cv. Ace without visible necrosis. Susceptible cvs. Earlypak-7 and VFN-Bush also consistently differed from each other, but both were clearly more sensitive to toxin than were cvs. Ace and Walter. Effects on resistant cultivars required treatment with 1000-fold greater

concentrations of toxin (12 µg/ml) than were required to induce necrosis in susceptible cultivars. A difference in toxin sensitivity greater than 1000-fold was evident when resistant cv. Walter was compared with susceptible cv. VFN-Bush. Nicotiana tabacum Wisconsin 38 tolerated 12 µg toxin/ml with no apparent damage.

Comparison of growth measurements for cell suspension cultures

Suspension cultures were established on media that supported optimal growth of each genotype. One resistant and one susceptible tomato cultivar were selected for optimal growth on each medium. Resistant cv. Walter and susceptible cv. Earlypak-7 were selected and maintained on culture medium R2A. Resistant cv. Ace and susceptible cv. VFN-Bush were selected and maintained on culture medium T-12. Tobacco was maintained on culture medium 3/.3.

Growth rates of cultures were compared by use of settled cell volume (98) and dry weight taken during logarithmic growth phase. The data were subjected to regression analyses. Growth rates and cell doublings/day were calculated. Comparable results were obtained from cell volume and dry weight data, as indicated by the high coefficients of determination (r^2) calculated from data for control (non-treated) cultures of all five genotypes (Table 1).

The original determination of the logarithmic growth phase for suspension cultures of each genotype was calculated from settled cell volume measurements. Discrepancies in the growth rates calculated from cell volume and dry weight data reflected asynchrony in the logarithmic growth phases determined from cell volume and dry weight data. Differences in growth measurements were also attributable to the inherent

Table 1. Growth Rates and Doublings of Suspension Cultures Per Day Determined by Settled Cell Volumes and Dry Weights of Cells.

Cultivar	Toxin ($\mu\text{g/ml}$)	Cell volume			r ²	Dry weights			
		a (ml)	b (ml/ml/hr)	doublings/ day		a (mg)	b (mg/mg/hr)	doublings/ day	
Earlypak-7 ^a	0	0.8805	4.5	0.0103	0.356	0.8028	53.1	0.0120	0.415
	0	0.9288	3.6	0.0111	0.386	0.9570	61.1	0.0125	0.432
	0	0.7948	4.1	0.0125	0.434	0.8390	49.0	0.0138	0.478
Ace ^b	0	0.9175	3.6	0.0104	0.360	0.9353	51.5	0.0099	0.342
	0	0.9414	3.8	0.0117	0.405	0.9332	60.8	0.0121	0.419
	0	0.7782	2.9	0.0139	0.482	-	-	-	-
Walter ^b	0	0.9342	4.3	0.0092	0.317	0.9658	73.4	0.0093	0.323
	40	0.9370	4.2	0.0038	0.132	0.9413	69.0	0.0035	0.122
	0	0.9516	3.2	0.0143	0.496	-	-	-	-
Tobacco ^c	4	0.9268	3.8	0.0088	0.305	-	-	-	-
	40	0.8335	3.6	0.0067	0.233	-	-	-	-
	400	0.8520	3.8	0.0059	0.206	-	-	-	-
	0	0.9113	4.4	0.0125	0.433	0.9496	53.0	0.0182	0.632
	40	0.9855	4.5	0.0098	0.340	0.9953	54.6	0.0157	0.543
	400	0.9984	4.3	0.0109	0.377	0.9980	52.1	0.0136	0.472
	4000	0.9687	4.5	0.0063	0.220	0.9224	56.0	0.0068	0.235
	0	0.9256	5.7	0.0120	0.416	0.9447	65.8	0.0149	0.516
20	0.9712	5.8	0.0105	0.364	0.9757	61.9	0.0140	0.483	
200	0.9148	6.0	0.0097	0.337	0.9694	58.9	0.0148	0.513	
2000	0.8459	5.9	0.0048	0.168	0.9517	57.6	0.0083	0.286	

^aSusceptible tomato genotype.

^bResistant tomato genotype.

^cNicotiana tabacum Wisconsin 38.

variability of suspension cultures.

Effects of toxin on growth of cell suspension cultures

Suspension cultures were initiated with 2 ml settled cell volume to which fresh medium was added to yield a total suspension volume of 50 ml. Toxin was added to suspension cultures at the end of the lag phase to give cultures with toxin concentrations of 200 ng/ml, 2 µg/ml, 20 µg/ml, 200 µg/ml, or 2 mg/ml. The toxin preparation used in the first experiment had specific activity at 11 ng/ml. Toxin with a specific activity at 12 ng/ml was used in other experiments; this preparation was added to cultures to give toxin concentrations of 400 ng/ml, 4 µg/ml, 40 µg/ml, 400 µg/ml, or 4 mg/ml.

Data were taken during the logarithmic growth phase. The data on settled cell volume and dry weight were subjected to regression analyses to describe growth of the suspension of cultures using the exponential curve fitting equation:

$$y = ae^{bx}.$$

Coefficients of determination, growth rates, and cell doublings per day were calculated from cell volume and dry weight data for all genotypes at all toxin treatments. High coefficients of determination (r^2) were obtained for control cultures of all genotypes. Tobacco suspension cultures treated with 20, 40, 200, 400 µg/ml, 2, or 4 mg/ml had high coefficients of determination. Suspension cultures of resistant cv. Walter treated with toxin concentrations of 4, 40, or 400 µg/ml also had high coefficients of determination (Table 1). High coefficients of determination confirmed that the cultures were in logarithmic growth phase during toxin treatment. Similar values of the extrapolated initial

cell volume or dry weight (a) of all treatments maintaining logarithmic growth confirmed the similarity of the replicates.

All coefficients of determination (r^2) calculated for toxin-treated suspension cultures of resistant cv. Ace and susceptible cvs. Earlypak-7 and VFN-Bush were very low, indicating that toxin interfered with logarithmic growth of these genotypes. Growth of suspension cultures of resistant cv. Walter treated with 4 mg toxin/ml was not logarithmic. Representative coefficients of determination, growth rates, and cell doublings per day calculated from settled cell volume data of all five genotypes are shown in Table 2. Settled cell volumes of toxin-treated suspension cultures could be distorted because of toxin-induced swelling, plasmolysis, and lysis.

Representative graphs of the regression curves calculated from the dependent variable (logarithm of settled cell volume) and the independent variable (time after toxin treatment) are presented in Figures 1-4. Corresponding standard errors of the means are presented in Tables 3-6. The data were adjusted to permit intercultural comparisons of growth rates. One-tailed t-tests were performed on growth rates calculated from adjusted settled cell volume data. Representative summaries of the t-tests are presented in Tables 7-10.

Growth rates of suspension cultures of all tomato genotypes were inhibited by treatment with toxin concentrations of 4 μ g/ml to 4 mg/ml. Growth rates of suspension cultures of tobacco were inhibited by treatment with toxin concentrations of 20 μ g/ml to 4 mg/ml. Growth rates of controls from all four genotypes differed significantly from growth rates of all toxin treatments at the 0.1% level, with one exception. No significant differences between growth rates of control and toxin-treated

Table 2. Growth Rates and Doublings of Suspension Cultures Per Day Determined by Settled Cell Volumes^a

Cultivar	Expt. No.	Toxin ($\mu\text{g/ml}$)	r^2	a (ml)	b (ml/ml/hr)	Doublings/ day
Earlypak-7 ^b	5	0	0.9288	3.6	0.0111	0.386
	5	40	0.1244	3.6	0.0010	0.033
	5	400	0.1888	3.7	0.0007	0.023
	5	4000	0.0225	3.7	0.0003	0.011
Walter ^c	5	0	0.9342	4.3	0.0092	0.317
	5	40	0.9370	4.2	0.0038	0.132
	5	400	0.7549	4.3	0.0017	0.059
	5	4000	0.1876	4.0	0.0010	0.034
Ace ^c	8	0	0.9175	3.6	0.0104	0.360
	8	40	0.4784	4.3	0.0035	0.122
	8	400	0.1654	4.2	0.0016	0.056
	8	4000	0.2207	4.1	0.0011	0.036
VFN-Bush ^b	8	0	0.9414	3.8	0.0117	0.405
	8	40	0.3286	3.9	0.0015	0.051
	8	400	0.1724	3.9	0.0009	0.032
	8	4000	0.1565	4.3	0.0011	0.037
Tobacco ^d	5	0	0.9113	4.4	0.0125	0.433
	5	40	0.9855	4.5	0.0098	0.340
	5	400	0.9984	4.3	0.0109	0.377
	5	4000	0.9687	4.5	0.0063	0.220

^aThese are representative data of controls and toxin-treated cell suspensions of all 5 genotypes.

^bSusceptible tomato genotype.

^cResistant tomato genotype.

^dN. tabacum Wisconsin 38.

Figure 1. Effect of toxin (0, 4, 40, 400 $\mu\text{g/ml}$) on growth of cell cultures of susceptible tomato cv. VFN-Bush, as determined by regression analyses of settled cell volume data.^a

^aRaw data and standard errors of the means are presented in Table 3. Summary of t-tests are presented in Table 9.

^bTime after toxin treatment.

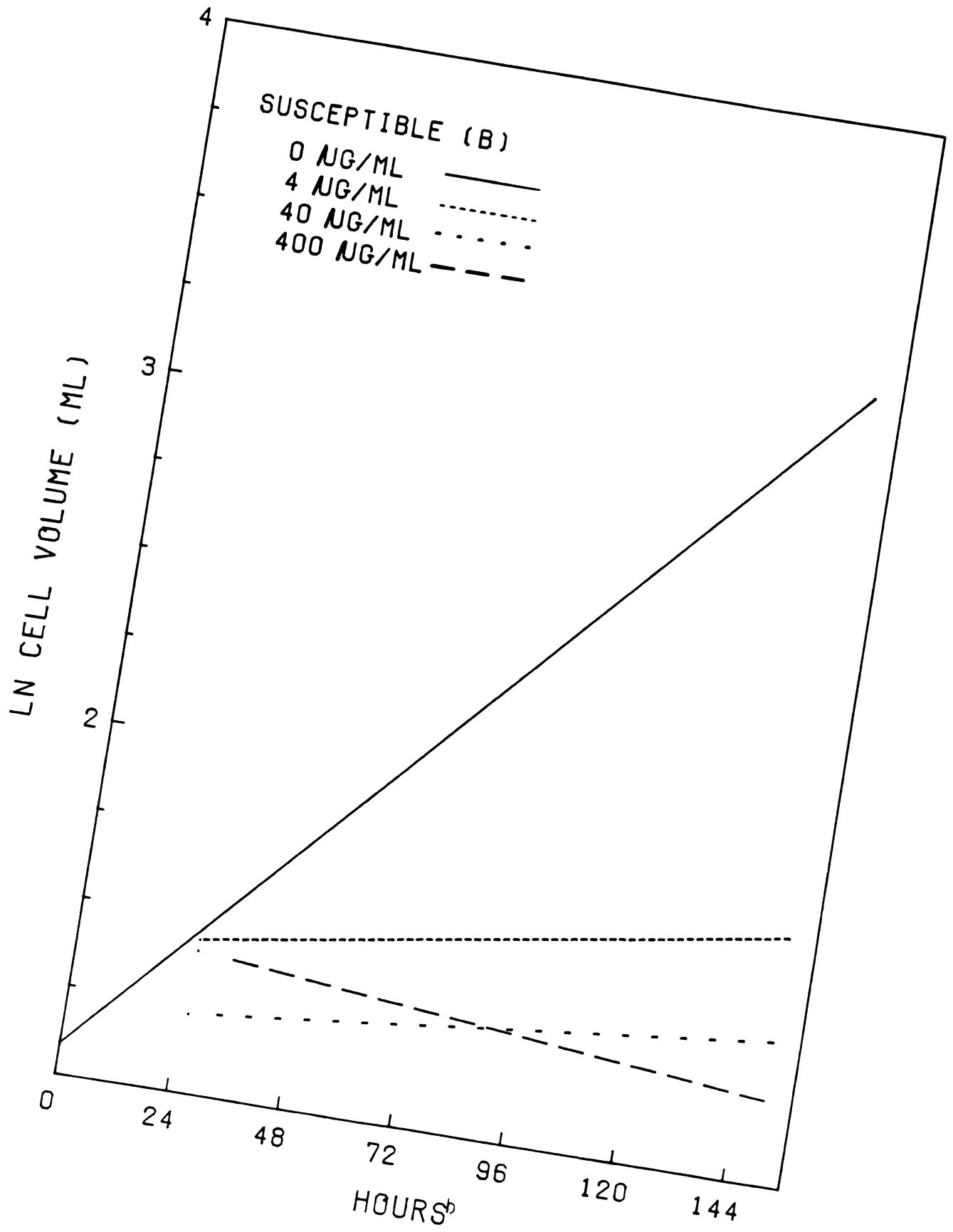


Figure 1.

Table 3. Effect of Toxin on Growth and Mortality of Suspension Cultures of Susceptible cv. VFN-Bush^a

<u>Toxin conc.</u> <u>(μg/ml)</u>	<u>Time^b</u> <u>(hr)</u>	<u>n</u>	<u>$\bar{X}$ Cell vol.^c</u> <u>(ml)</u>	<u>S.E.^d</u>	<u>$\bar{X}$ Dead^e</u> <u>(%)</u>
0	0	3	4.7	0.1	-
	26	8	3.9	0.3	6
	51	8	5.4	0.4	4
	97	9	10.6	1.6	4
	150	8	29.2	4.1	6
4	26	3	3.8	0.2	10
	51	3	4.6	0.2	19
	97	3	4.7	0.4	35
	150	3	5.3	1.2	49
	26	3	3.5	0.3	20
40	51	3	3.3	0.2	36
	97	3	3.4	0.6	47
	150	2	4.2	0.2	51
	26	3	3.5	0.3	25
	51	3	4.1	0.3	39
400	97	3	4.2	0.6	58
	150	2	2.9	0.1	68

^aExperiment was repeated twice with comparable results.

^bTime after toxin treatment.

^c $\bar{X}$ Volume of cells settled for 30 min.

^dStandard error of the mean.

^e $\bar{X}$ Dead (%) of 2 determinations (bromophenol blue stain) from one sample/treatment.

Figure 2. Effect of toxin (0, 4, 40, 400 $\mu\text{g/ml}$) on growth of cell cultures of resistant tomato cv. Walter, as determined by regression analyses of settled cell volume data.^a

^aRaw data and standard errors of the means are presented in Table 4. Summary of t-tests are presented in Tables 7 and 8.

^bTime after toxin treatment.

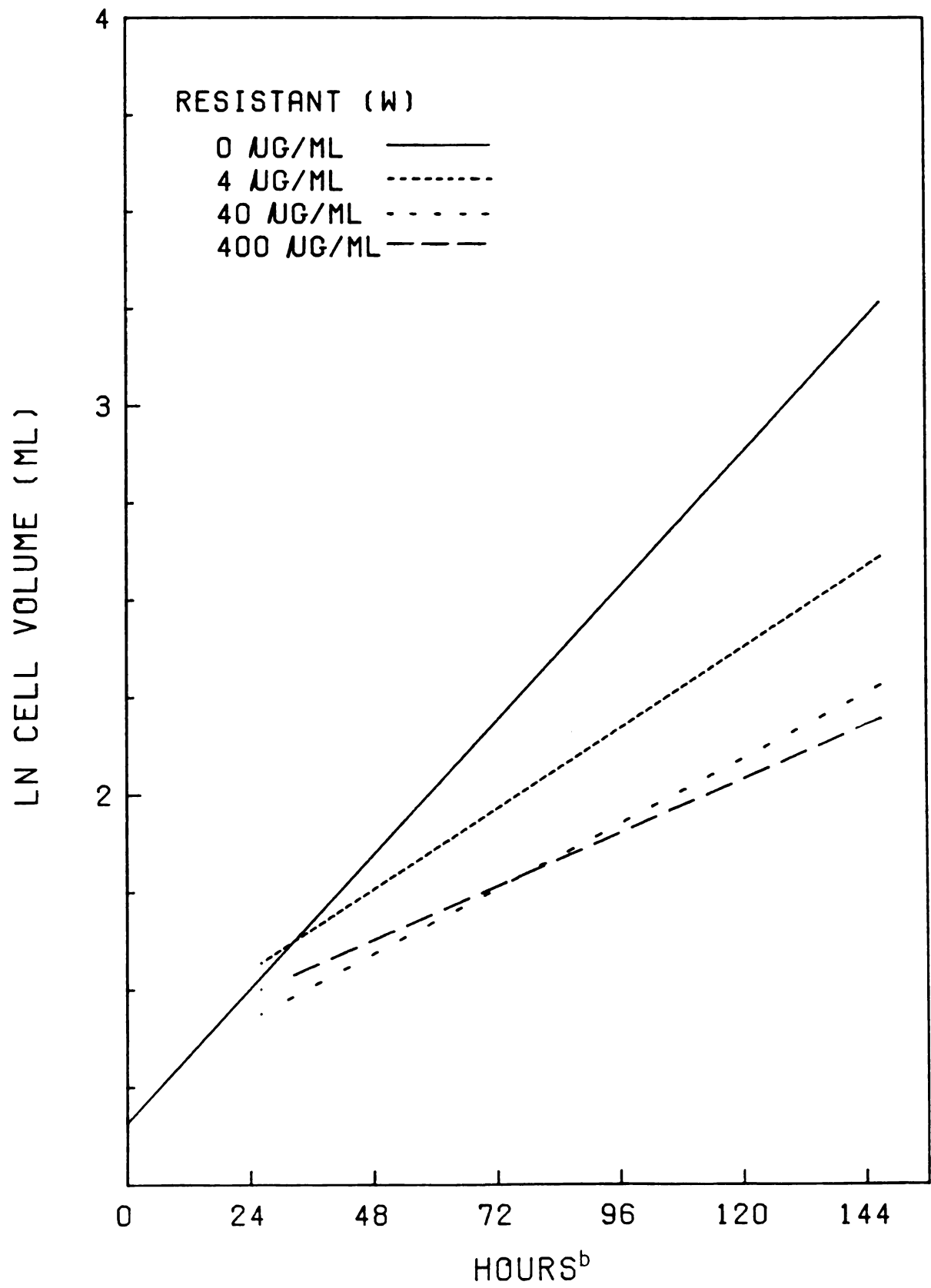


Figure 2.

Table 4. Effect of Toxin on Growth and Mortality of Suspension Cultures of Resistant cv. Walter^a

<u>Toxin conc.</u> ($\mu\text{g/ml}$)	<u>Time^b</u> (hr)	<u>n</u>	<u>X Cell vol.^c</u> (ml)	<u>S.E.^d</u>	<u>X Dead^e</u> (%)
0	0	3	3.7	0.4	-
	26	9	4.3	0.1	7
	50	9	6.6	0.2	4
	96	9	13.6	1.2	2
	147	10	26.1	1.1	4
4	26	3	5.1	0.5	10
	50	3	5.3	0.4	7
	96	3	9.5	0.5	11
	147	3	13.3	0.7	11
40	26	3	3.9	0.3	7
	50	3	5.3	0.8	10
	96	3	7.5	0.3	13
	147	3	9.3	1.1	17
400	26	3	4.2	0.2	6
	50	3	5.1	0.3	8
	96	3	8.0	0.3	11
	147	3	8.2	0.4	18

^aExperiment was repeated once with comparable results.

^bTime after toxin treatment.

^cX Volume of cells settled for 30 min.

^dStandard error of the mean.

^eX Dead (%) of 2 determinations (bromophenol blue stain) from one sample/treatment.

Figure 3. Effect of toxin (0, 20, 200, 2000 $\mu\text{g/ml}$) on growth of cell cultures of resistant tomato cv. Ace, as determined by regression analyses of settled cell volume data.^a

^aRaw data and standard errors of the means are presented in Table 5. Summary of t-tests are presented in Table 10.

^bTime after toxin treatment.

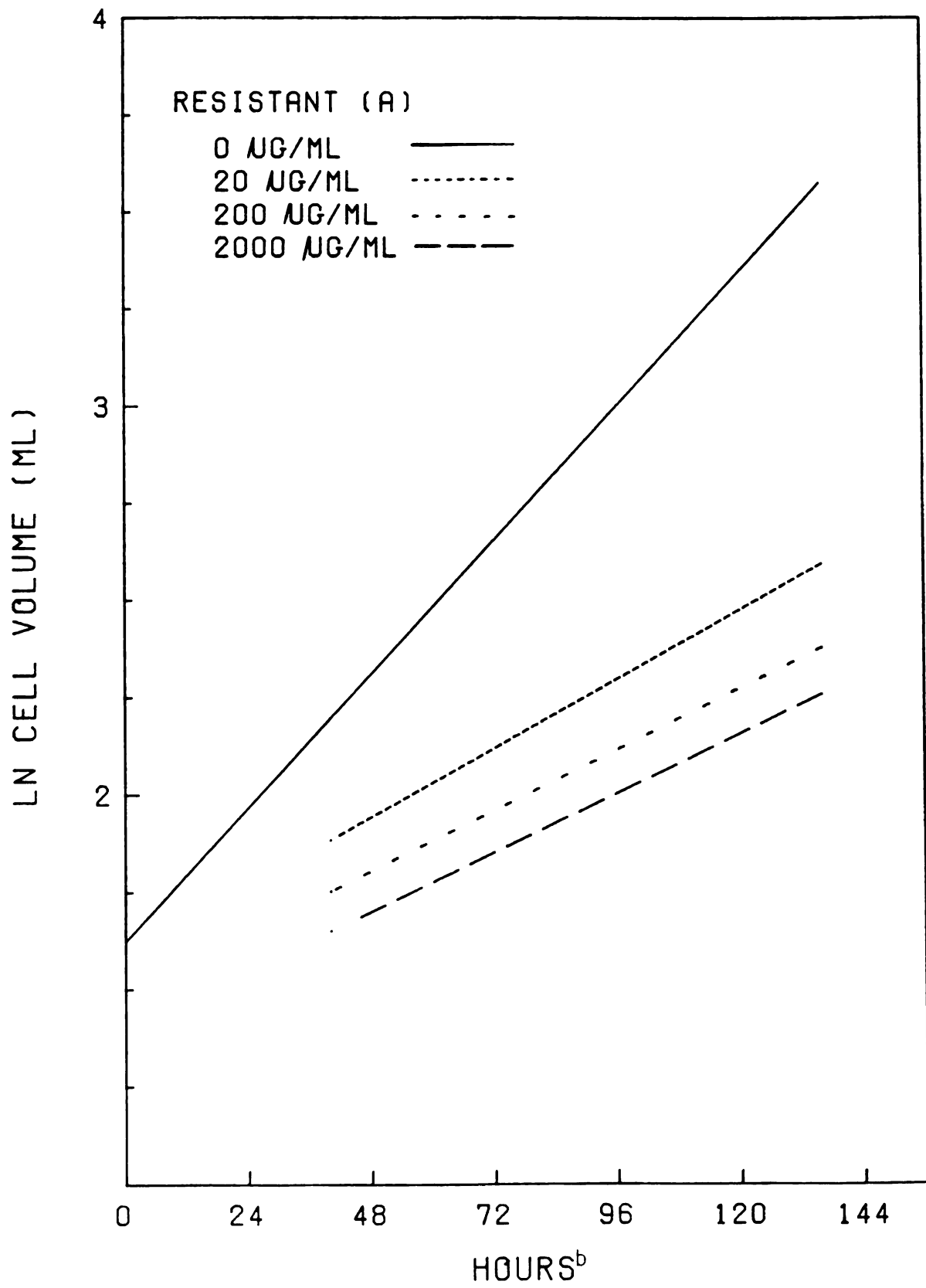


Figure 3.

Table 5. Effect of Toxin on Growth and Mortality of Suspension Cultures of Resistant cv. Ace

Toxin conc. ($\mu\text{g/ml}$)	Time ^a (hr)	n	$\bar{X}$ Cell vol. ^b (ml)	S.E. ^c	$\bar{X}$ Dry wt (mg)	S.E. ^c	$\bar{X}$ Dead ^d (%)
0	26	9	5.1	0.2	68.43	3.16	7
	48	9	5.6	0.2	80.11	2.92	5
	96	9	10.1	0.5	137.80	4.52	6
	122	11	13.2	0.5	169.97	3.88	7
40 ^e	26	3	4.9	0.5	57.90	5.16	11
	48	3	5.3	0.6	66.90	5.20	11
	96	3	6.9	0.5	89.27	4.00	16
	122	3	6.0	0.3	80.70	3.20	15
400 ^e	26	3	4.9	0.3	58.13	5.61	12
	48	3	4.0	0.0	54.27	0.87	20
	96	3	5.9	0.5	74.53	5.16	32
	122	3	4.6	0.1	69.50	2.28	28
4000 ^e	26	3	4.6	0.3	55.70	5.92	14
	48	3	4.2	0.2	57.17	1.98	22
	96	3	4.8	0.2	66.97	6.18	36
	122	3	4.5	0.0	61.90	1.76	32
0	0	3	5.0	0.4	57.15	2.97	17
	40	3	5.1	0.9	65.63	13.92	14
	88	3	14.5	2.2	199.17	15.48	9
	136	3	24.5	6.1	334.17	80.56	3
20 ^f	40	3	5.0	0.6	58.30	6.84	21
	88	3	8.4	2.1	91.23	10.04	22
	136	3	9.9	2.8	114.73	21.34	51
	40	3	4.4	0.6	55.60	5.70	28
200 ^f	88	3	7.2	0.9	103.30	21.84	37
	136	2	7.2	1.2	69.05	11.14	43
	40	3	4.4	1.1	49.80	1.21	17
	88	2	6.0	1.0	79.55	5.75	53
2000 ^f	136	4	7.1	0.5	79.23	3.54	60

^aTime after toxin treatment.^b $\bar{X}$ Volume of cells settled for 30 min.^cStandard error of the mean.^d $\bar{X}$ dead (%) of 2 determinations (bromophenol blue stain) from one sample/treatment.^eToxin preparation spec. activity 12 ng/ml.^fToxin preparation spec. activity 11 ng/ml.

Figure 4. Effect of toxin (0, 40, 400, 4000 $\mu\text{g/ml}$) on growth of cell cultures of susceptible tomato cv. Earlypak-7, as determined by regression analyses of settled cell volume data.^a

Note: Raw data and standard errors of the means are presented in Table 6. Summary of t-tests are presented in Table 8.

^bTime after toxin treatment.

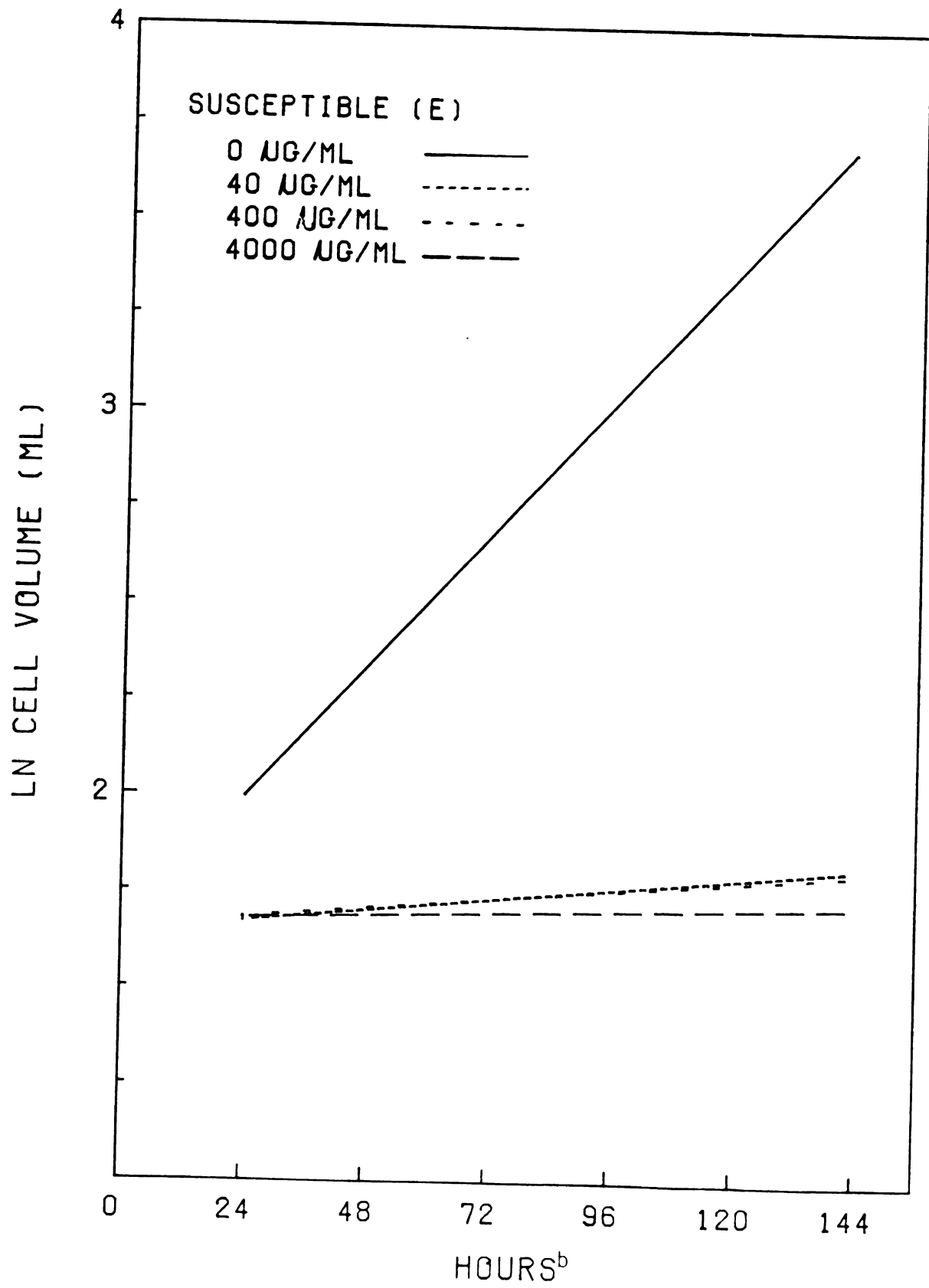


Figure 4.

Table 6. Effect of Toxin on Growth and Mortality of Suspension Cultures of Susceptible cv. Earlypak-7^a

<u>Toxin conc.</u> <u>(μg/ml)</u>	<u>Time^b</u> <u>(hr)</u>	<u>n</u>	<u>$\bar{X}$ Cell vol.^c</u> <u>(ml)</u>	<u>S.E.^d</u>	<u>$\bar{X}$ Dry wt</u> <u>(mg)</u>	<u>S.E.^d</u>	<u>$\bar{X}$ Deade</u> <u>(%)</u>
0	24	8	4.8	0.2	81.7	1.8	4
	50	8	6.5	0.3	110.7	5.7	5
	72	9	7.8	0.5	161.5	7.6	7
	142	9	18.0	1.0	354.5	11.0	9
40	24	3	3.8	0.2	64.9	3.0	21
	50	3	3.9	0.3	72.1	7.8	50
	72	3	3.4	0.1	68.7	6.2	64
	142	3	4.3	0.4	67.6	4.0	89
400	24	3	3.9	0.1	67.7	0.7	27
	50	3	3.8	0.2	68.5	4.5	64
	72	3	3.7	0.2	69.4	5.7	77
	142	3	4.2	0.2	59.9	10.0	100
4000	24	3	3.7	0.2	63.1	1.7	38
	50	3	3.8	0.4	61.1	9.3	79
	72	3	3.7	0.2	57.5	4.2	90
	142	3	3.9	0.3	62.8	6.2	100

^aExperiment was repeated once with comparable results.

^bTime after toxin treatment.

^c $\bar{X}$ Volume of cells settled for 30 min.

^dStandard error of the mean.

^e $\bar{X}$ Dead (%) of 2 determinations (bromophenol blue stain) from one sample/treatment.

Table 7. Summary of One-tailed T-tests of Growth Rates of Suspension Cultures of Resistant cv. Walter and Susceptible cv. VFN-Bush^a

		Walter				VFN-Bush		
		0	4	40	400	0	4	40 ^b
Walter	4	5.100 48 df ***						
	40	6.285 48 df ***	1.200 20 df n.s.					
	400	7.672 48 df ***	2.395 20 df *	0.885 20 df n.s.				
VFN-Bush	4		4.588 19 df ***			4.483 43 df ***		
	40			3.332 19 df **		4.714 43 df ***	0.399 18 df n.s.	
	400				4.539 19 df ***	5.707 43 df ***	1.955 18 df n.s.	1.521 18 df n.s.

^aData were adjusted to equalize growth rates of controls of both genotypes. Growth rates were calculated from settled cell volume data.

^b μ g toxin/ml

Significance levels: * = 0.05
 ** = 0.01
 *** = 0.001

Table 8. Summary of One-tailed T-tests of Growth Rates of Suspension Cultures of Resistant cv. Walter and Susceptible cv. Earlypak-7^a

		Walter				Earlypak-7		
		0	4	40	400	0	40	400 ^b
Walter	4	5.100 48 df ***						
	40	6.285 48 df ***	1.200 20 df n.s.					
	400	7.672 48 df ***	2.395 20 df *	0.885 20 df n.s.				
Earlypak-7	40			3.639 20 df **		9.302 42 df ***		
	400				4.258 19 df ***	9.996 41 df ***	0.148 19 df n.s.	
	4000		9.140 20 df ***			10.328 42 df ***	0.621 20 df n.s.	0.617 19 df n.s.

^aData were adjusted to equalize growth rates of controls of both genotypes. Growth rates were calculated from settled cell volume data.

^bµg toxin/ml

Significance levels: * = 0.05
 ** = 0.01
 *** = 0.001

Table 9. Summary of One-tailed T-tests of Growth Rates of Suspension Cultures of Resistant cv. Ace and Susceptible cv. VFN-Bush^a

		Ace				Bush		
		0	40	400	4000	0	40	400 ^b
Ace	40	5.398 46 df ***						
	400	7.631 47 df ***	1.352 19 df n.s.					
	4000	9.067 47 df ***	2.114 19 df *	0.347 20 df n.s.				
Bush	40		1.601 19 df n.s.			10.037 43 df ***		
	400			0.235 21 df n.s.		11.180 44 df ***	0.558 21 df n.s.	
	4000				0.055 21 df n.s.	11.180 44 df ***	0.751 21 df n.s.	0.240 22 df n.s.

^aData were adjusted to equalize growth rates of controls of both genotypes. Growth rates were calculated from settled cell volume data.

^b μ g toxin/ml

Significance levels: * = 0.05
 ** = 0.01
 *** = 0.001

Table 10. Summary of One-tailed T-tests of Growth Rates of Suspension Cultures of Resistant cv. Ace and Susceptible cv. Earlypak-7^a

		Ace				Earlypak-7		
		0	20	200	2000	0	20	200 ^b
Ace	20	1.613 17 df n.s.						
	200	1.952 16 df n.s.	0.181 13 df n.s.					
	2000	2.261 17 df *	0.239 14 df n.s.	0.048 13 df n.s.				
Earlypak-7	20		1.141 14 df n.s.			4.385 17 df ***		
	200			3.039 13 df **		6.519 17 df ***	2.105 14 df n.s.	
	2000				3.553 14 df **	6.929 17 df ***	2.718 14 df *	1.121 14 df n.s.

^aData were adjusted to equalize growth rates of controls of both genotypes. Growth rates were calculated from settled cell volume data.

^b μ g toxin/ml

Significance levels: * = 0.05
 ** = 0.01
 *** = 0.001

cultures (20, 200 $\mu\text{g/ml}$) of the resistant cv. Ace were detected in the initial experiment. The growth rate of suspensions of resistant cv. Ace exposed to 2 mg toxin/ml differed significantly from the growth rate of the non-treated cultures at the 5% level (Table 10).

Growth rates of toxin-treated suspension cultures of resistant cv. Walter were significantly higher (1% or 0.1% level) than growth rates of comparably treated suspension cultures of susceptible cvs. Earlypak-7 and VFN-Bush at all toxin concentrations in all experiments (Tables 7 and 8). The growth rates of suspension cultures of resistant cv. Ace treated with toxin concentrations of 200 $\mu\text{g/ml}$ and 2 mg/ml differed from comparably treated suspension cultures of susceptible cv. Earlypak-7 (1% level) (Table 10). Growth rates of suspension cultures of resistant cv. Ace were highly variable; they did not differ significantly from growth rates of either susceptible cultivar exposed to toxin at 40 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$ and 4 mg/ml (Table 9). The growth rates of all tomato cultivars exposed to toxin concentrations of 200 ng/ml and 2 $\mu\text{g/ml}$ were highly variable; the data are not presented. T-tests were not used on the growth data of tobacco cultures because there was insufficient replication.

Growth rates of suspension cultures of resistant cv. Walter treated with toxin concentrations that differed by a factor of 100 were significantly different from each other at the 5% level (Table 8). The same was true in one experiment (Table 9) with the resistant cv. Ace, but results were not confirmed in a second experiment (Table 10). Growth rates of toxin-treated suspension cultures of susceptible tomato cultivars did not differ significantly from each other at any toxin concentration.

Mortality of toxin-treated and control cells

Mortality was determined by staining the cells with bromphenol blue, with the assumption that live cells exclude the stain. Two determinations were averaged to obtain the mean percentage of dead cells in a population of 500 cells for each genotype at each toxin treatment and time of determination. Representative samples from cultures of cells that excluded the bromophenol blue stain were stained with fluorescein diacetate to confirm viability. Live cells contain esterases which cleave the acetate residues and permit accumulation of fluorescein. Cells containing fluorescein are fluorescent when viewed under dark-field microscopy using exciter filters and barrier filters (83).

Control (non-treated) cultures of all genotypes had a similar percentage of dead cells. Toxin caused cell death in all genotypes at all concentrations of toxin (200 $\mu\text{g/ml}$ to 4 mg/ml). Differences in the percentages of toxin-induced cell death were observed between genotypes at all concentrations of toxin treatment. Resistant cvs. Walter and Ace had fewer dead cells in all toxin-treated cultures than did comparably treated cultures of susceptible cvs. Earlypak-7 and VFN-Bush. Fewer cells were killed in resistant cv. Walter than in resistant cv. Ace in comparable toxin treatments. Susceptible cv. VFN-Bush had fewer dead cells than susceptible cv. Earlypak-7 in comparable toxin treatments. Representative graphs of the percentages of toxin-induced cell death in all four tomato genotypes are given (Figures 5 and 6). Tobacco cultures showed little or no toxin-induced cell death at all toxin concentrations tested (20 $\mu\text{g/ml}$ to 4 mg/ml). The degree of toxin-induced cell death was a genotype-specific phenomenon that increased in proportion to the log of the toxin concentration and the total time of exposure to toxin.

Figure 5. Percentage of toxin-induced cell death in suspension cultures of resistant cv. Walter and susceptible cv. Earlypak-7.

^aTime after toxin treatment.

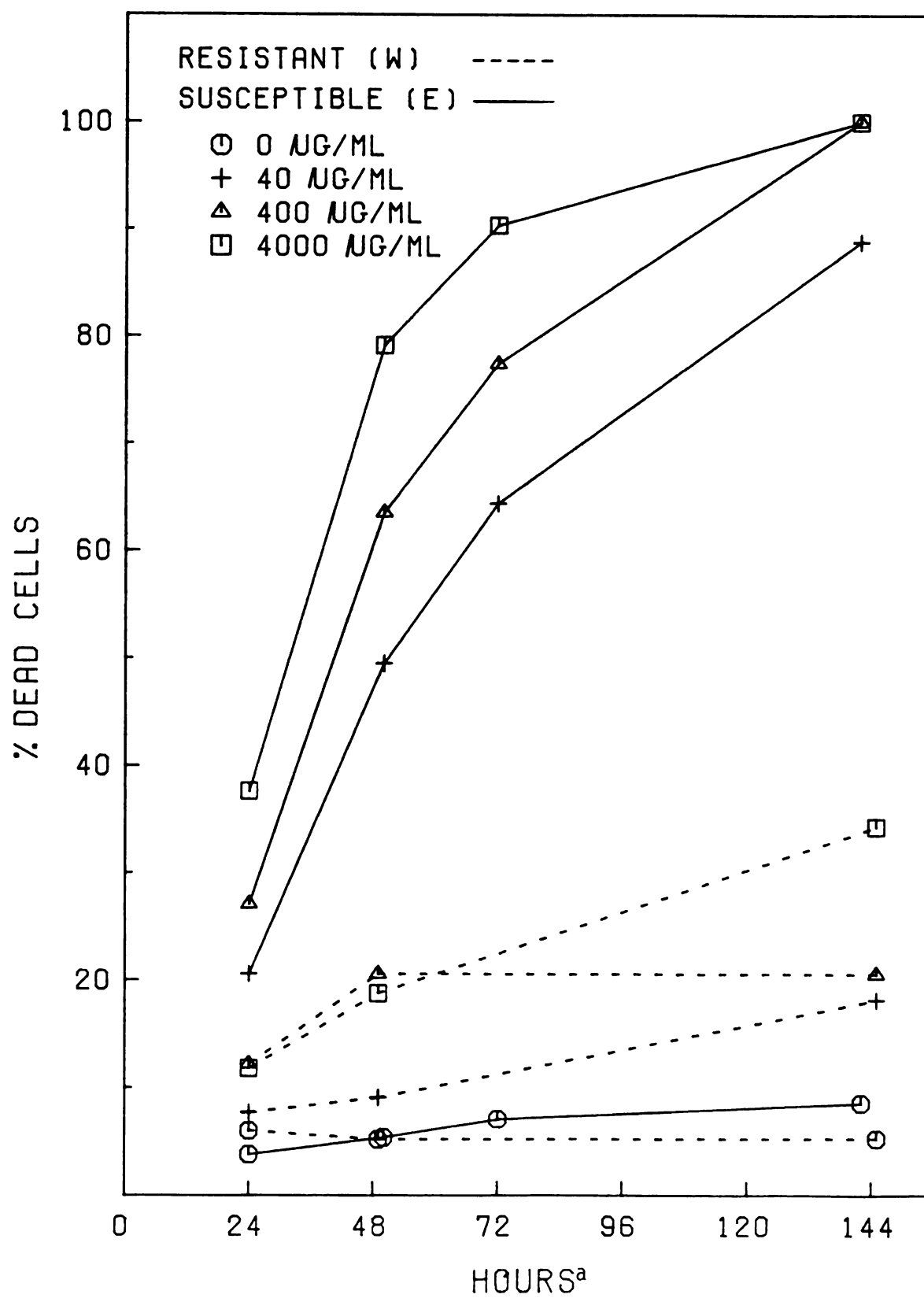


Figure 5.

Figure 6. Percentage of toxin-induced cell death in suspension cultures of resistant cv. Ace and susceptible cv. VFN-Bush.

^aTime after toxin treatment.

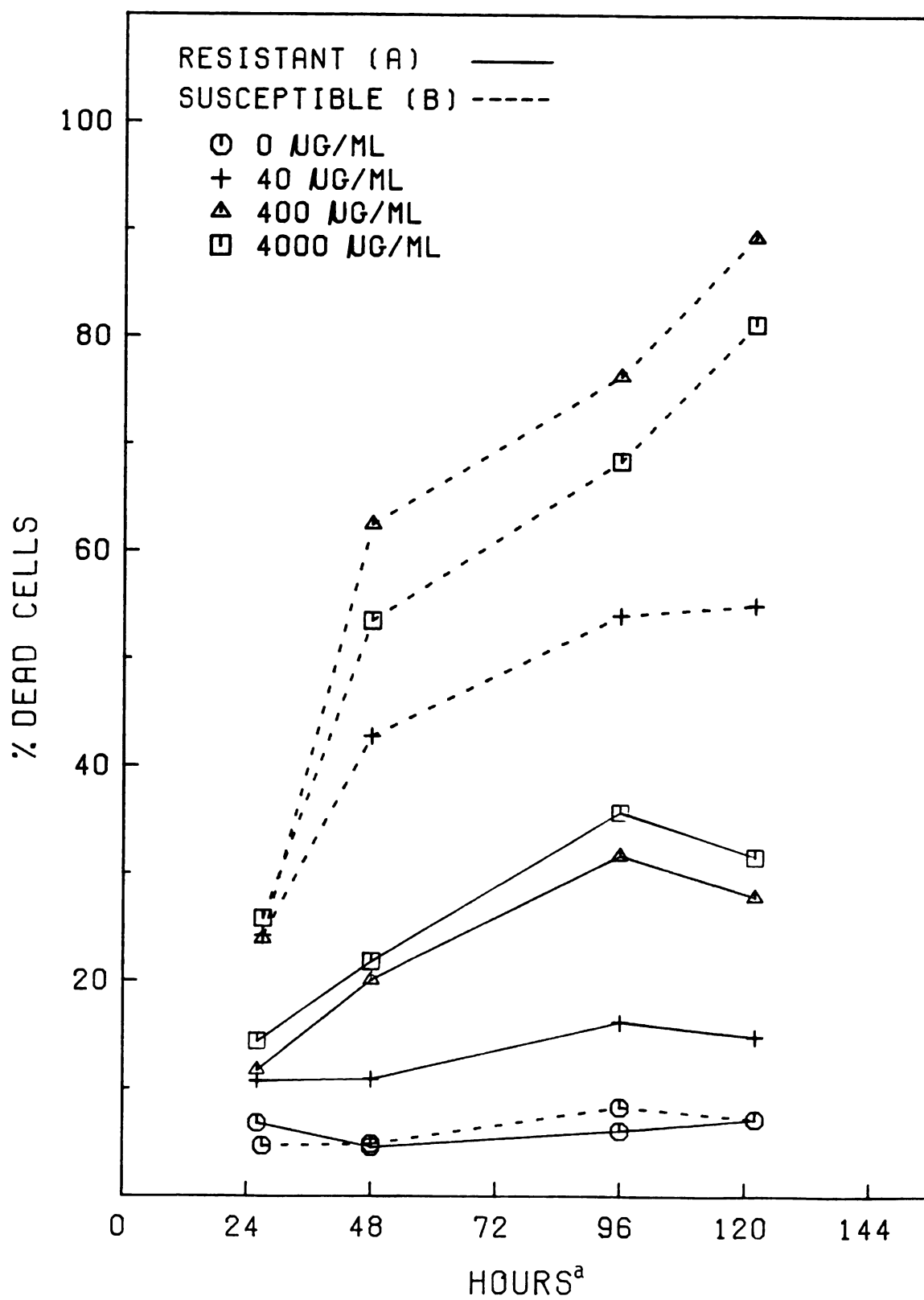


Figure 6.

Toxin-induced discoloration of suspension cultures

All concentrations of toxin (200 ng/ml to 4 mg/ml) caused dramatic discoloration of susceptible but not resistant cells and supernatant liquids. Discoloration was detected within 24 hours of toxin treatment, and increased in intensity throughout the experiments. Susceptible cv. Earlypak-7 progressed from normally beige cells and colorless supernatant liquid to rust, brown and black cells and supernatant liquids. Susceptible cv. VFN-Bush progressed from normal beige suspensions to charcoal grey and black cells and supernatant liquids. Control suspension cultures were not discolored at any time during the experiments. Resistant tomato genotypes and tobacco suspension cultures were only slightly discolored when exposed to the highest toxin concentrations (2 mg/ml, 4 mg/ml), but the discoloration corresponded to the color of the toxin preparation that was added to the suspension cultures. Discoloration in the susceptible genotypes was apparently due to accumulation of polymerized phenolic substances, but the phenomenon was difficult to quantitate because components of the culture medium interfered with spectrophotometric examination. Determination of total phenolics by Prussian blue colorimetric assay (96), with chlorogenic acid standards, was unsatisfactory. It appeared that polymerization of phenolic substances increased with the log of the toxin concentration, therefore providing fewer reactive sites for colorimetric reactions. Toxin-induced discoloration of suspension cultures was a genotype-specific phenomenon. The degree of discoloration was affected by toxin concentration and the total time of exposure to toxin.

Spectrophotometric and chromatographic examination of filtrates of toxin-treated suspension cultures

Cell suspension cultures that had been exposed to toxin at concentrations of 0, 40, 400 or 4000 $\mu\text{g/ml}$ for 120 hours were filtered, frozen, and stored for two months; after thawing, the supernatant liquids were concentrated five-fold under reduced pressure. The preparations were scanned on a single beam spectrophotometer at wavelengths of 640 to 200 nm. The toxin solution (20 $\mu\text{g/ml}$, pH 6.0) (Figure 8) and the supernatant solution from susceptible cv. Earlypak-7 had no absorption at these wavelengths. Comparable supernatant preparations from cultures of the resistant cv. Walter which had been treated with toxin at concentrations of 40 or 400 $\mu\text{g/ml}$ had an absorption peak at 318 nm (Figure 7). Supernatant solutions from cultures of the resistant cv. Walter which had been treated with toxin at 4 mg/ml had an absorption peak at 342 nm, rather than at 318 nm.

Aliquots of freshly harvested cell-free aqueous filtrates from a second experiment were extracted with ethyl acetate and the extract was dissolved in methanol. Toxin concentrations in the extracts were the same as the toxin concentrations used to treat the suspension cultures (400 ng/ml to 4 mg/ml). Absorption between 400 and 200 nm was monitored. Methanolic solutions of resistant cv. Walter gave absorption peaks at 228, 293, and 318 nm (Figure 9). The changes in absorbance increased in proportion to the log of the toxin concentration. Extracts from control (no toxin) cultures gave similar but diminished absorbance at these wavelengths. Comparable extracts of susceptible cv. Earlypak-7 did not absorb in these wavelengths (Figure 10).

Preparations which had been examined spectrophotometrically were

Figure 7. UV-absorption spectra of filtrates from cultures of resistant cv. Walter which had been treated with 40, 400, or 4000 μg toxin/ml. Absorption of the control filtrates was subtracted from the absorption of each toxin-treated filtrate. Bottom curve is for 40 μg toxin; top curve is for 4000 μg toxin.

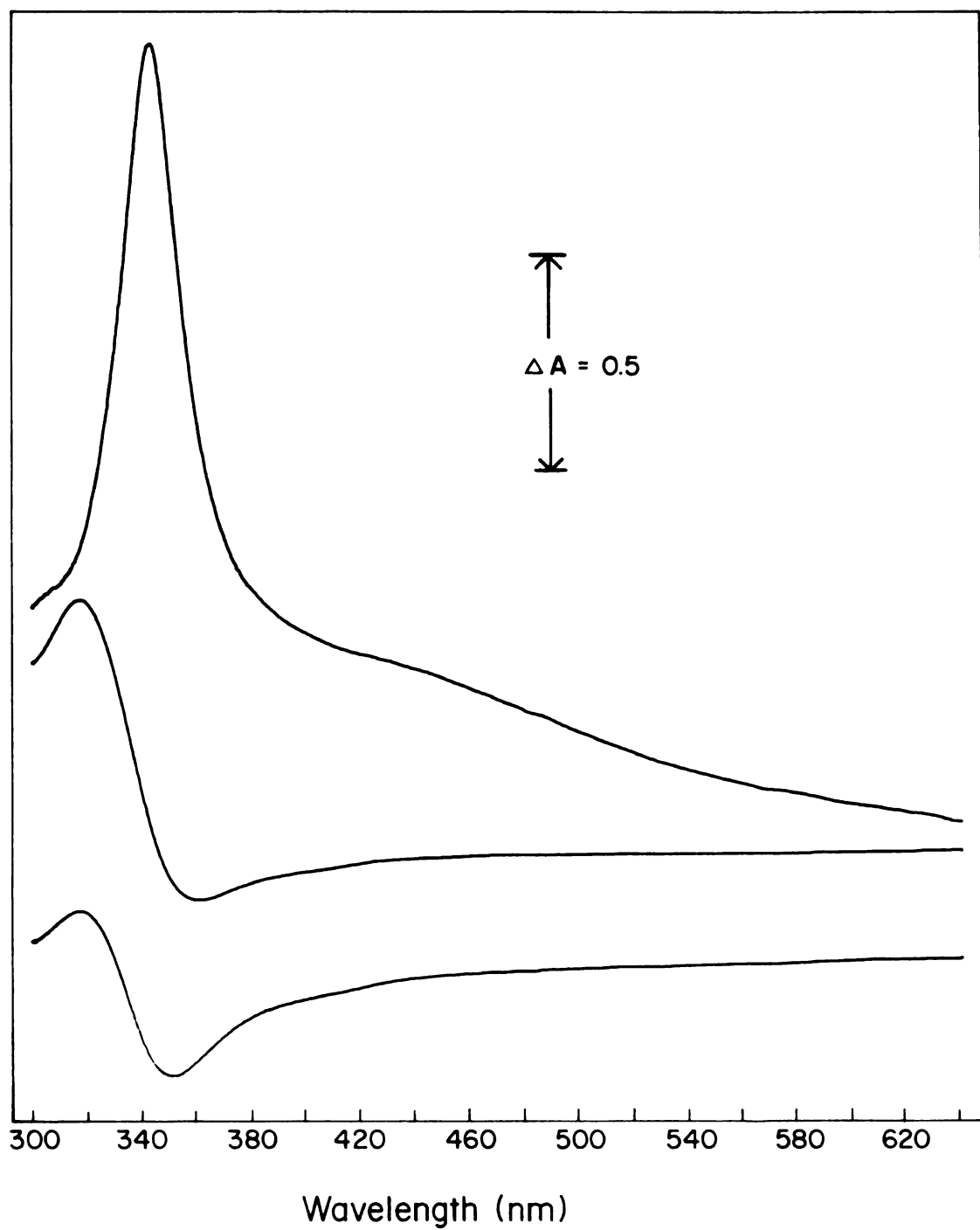


Figure 7.

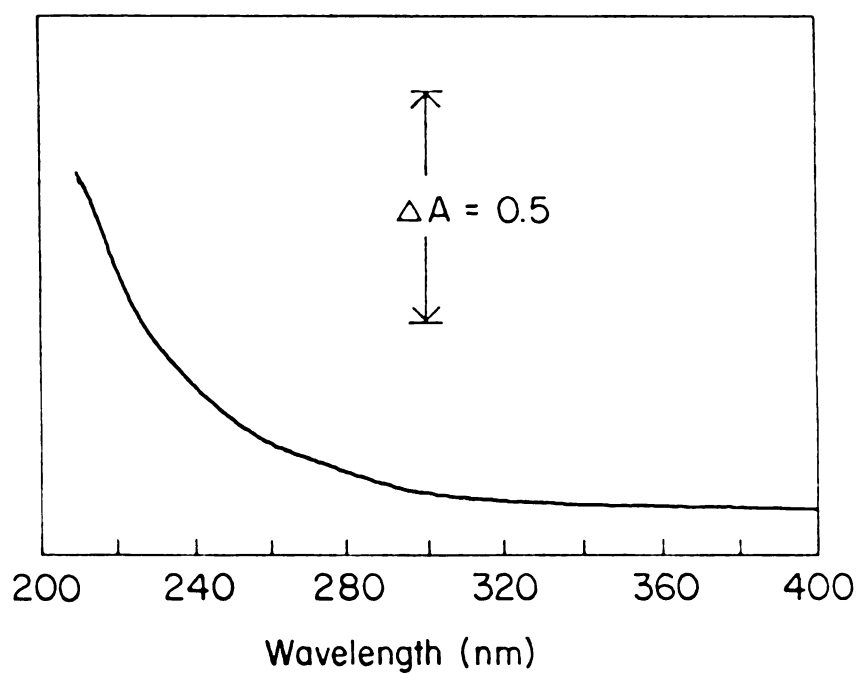


Figure 8. UV-absorption spectrum of aqueous toxin preparation (20 $\mu\text{g/ml}$, pH 6.0).

Figure 9. UV-absorption spectra of methanolic extracts from cultures of resistant cv. Walter which had been treated with toxin at 0, 0.4, 4, 40, 400, or 4000 $\mu\text{g}/\text{ml}$ (bottom to top).^a

^aAbsorption of the methanol solvent has been subtracted from each spectrum.

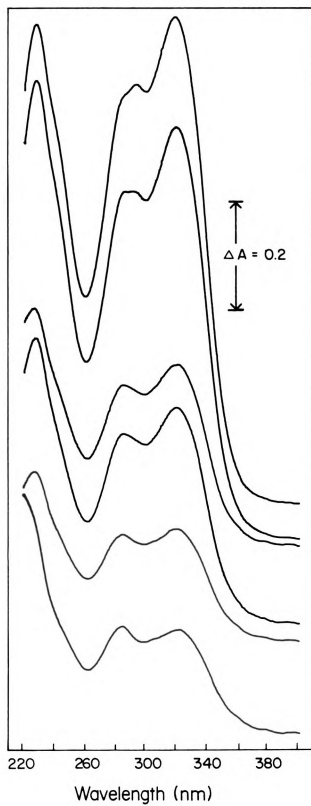


Figure 9.

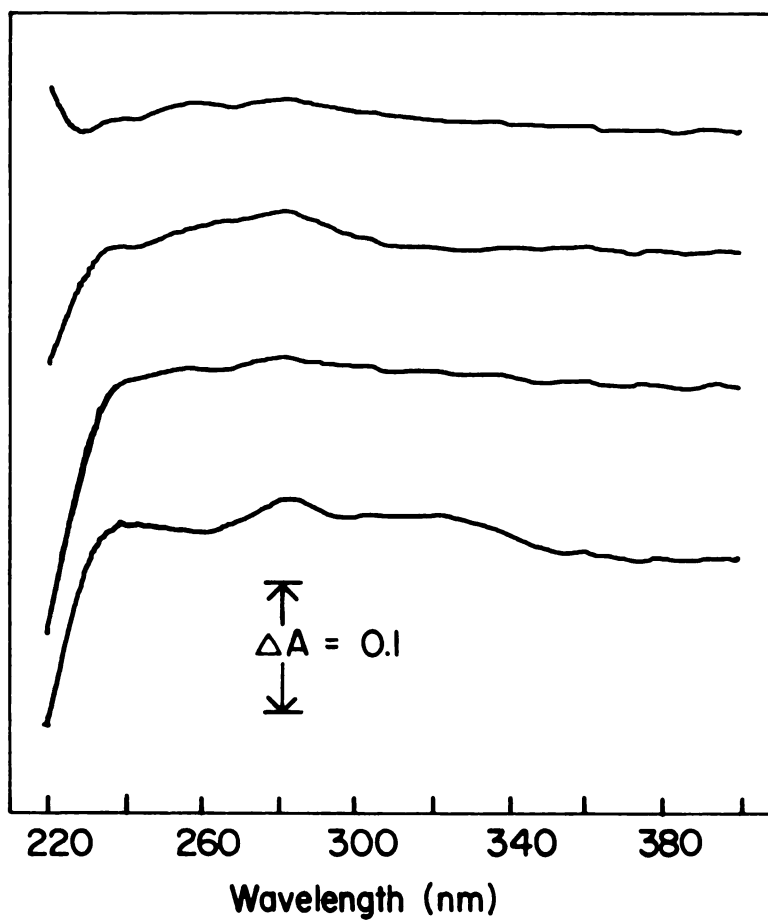


Figure 10. UV-absorption spectra of methanolic extracts from cultures of susceptible cv. Earlypak-7 which had been treated with toxin at 0, 40, 400, or 4000 $\mu\text{g/ml}$ (bottom to top).^a

^aAbsorption of the methanol solvent has been subtracted from each spectrum.

chromatographed on paper in an attempt to separate and identify the UV-absorbing substances. Droplets (10 μ l) of preparations were spotted and developed with acetic acid:water (15:85, v:v) or 1-propanol:ammonium hydroxide (7:3, v:v). Droplets (5 μ l) of marker compounds (chlorogenic acid, p-coumaric acid, ferulic acid, and caffeic acid) were chromatographed with the samples. Chromatograms were viewed with long and short wave UV-light. Resolution obtained on paper chromatograms was generally poor. Rf values of the standards did not correspond to Rf values of the unknowns in either solvent system. Discrete resolution of unknown compounds was not achieved.

Droplets (10 μ l) of the preparations in methanol were applied to TLC plates and developed with chloroform:methanol (95:5, v:v). Rishitin solution (5 μ l droplets) was cochromatographed as a standard. The chromatogram was viewed with UV-light, then sprayed with concentrated sulfuric acid. Rishitin, the only compound detected with sulfuric acid spray, had an Rf value of 0.18. Five poorly separated UV-fluorescing areas were observed in preparations from the resistant cv. Walter treated with 40, 400 or 4000 μ g toxin/ml. The spots had Rf values of 0.02, 0.05, 0.08, 0.15, and 0.22. Samples from control cultures of resistant cv. Walter gave spots with Rf values of 0.02, 0.03, 0.05, and 0.13. Similarly prepared samples from the non-treated control cultures of susceptible cv. Earlypak-7 had two UV-fluorescing spots with Rf values of 0.03 and 0.05.

Improved resolution was achieved by developing TLC plates in chloroform:methanol (9:1, v:v). Still better resolution was achieved by developing TLC plates in chloroform:methanol:water (65:25:4, v:v:v), drying the plate, and redeveloping the plate in the same solvent system.

Eleven distinct UV-fluorescing spots were detected in extracts of cultures of resistant cv. Walter treated with toxin concentrations of 40, 400, or 4000 $\mu\text{g/ml}$. Spots with Rf values of 0.47, 0.58, 0.61, 0.62, 0.67, 0.75, 0.81, and 0.83 were visible in control and treated extracts. In addition, spots with Rf values 0.38, 0.42, and 0.54 were readily detected in toxin-treated samples. The intensity of the UV-fluorescing spots appeared to be enhanced in proportion to the log of the toxin concentration. Composite spectra of the UV-absorbing compounds are shown in Figure 9. The change in absorbance appeared to increase in proportion to the log of the toxin concentration.

DISCUSSION

Growth rates of suspension cultures of all tomato genotypes were inhibited by treatment with toxin concentrations of 4 $\mu\text{g/ml}$ to 4 mg/ml . Growth rates of suspension cultures of tobacco were inhibited by treatment with toxin concentrations of 20 $\mu\text{g/ml}$ to 4 mg/ml . Although all genotypes exhibited toxin-induced growth inhibition, genotype-specific differential sensitivity to toxin was expressed in the relative amount of toxin-induced inhibition of growth. Suspension cultures of resistant tomato genotypes and tobacco tolerated 100-fold greater toxin concentrations with less growth inhibition than did susceptible genotypes. Growth rates of suspension cultures of resistant tomato genotypes treated with toxin concentrations that differed by a factor of 100 differed significantly from each other. Thus, it appeared that the relative amount of toxin-induced growth inhibition was genotype-specific and varied with respect to toxin concentration and the total exposure time.

Cvs. Walter, Earlypak-7 and VFN-Bush were consistent in their growth responses to toxin. The responses correlated with the effects of toxin on leaves and with relative resistance or susceptibility to the pathogen. In contrast, cultures of cv. Ace showed erratic growth responses to toxin; there was no conclusive correlation with responses of leaflets to toxin and of plants to the pathogen. There was much variation in toxin sensitivity of suspension cultures of cv. Ace.

There may be several explanations for the variability of cv. Ace.

Cumulative cell doublings of toxin-treated cells of cv. Ace in one experiment increased from 0 to 96 hours after toxin treatment, but decreased between 96 and 122 hours, at which time the experiment was terminated. This suggests that something occurred between 96 and 122 hours in all toxin-treated cultures to interfere with cell growth. Growth in control cultures, as measured by cumulative cell doublings, did not decrease over this time period. Accumulation of cytotoxic products related to toxin, to detoxification, or to an unrelated event to which toxin-treated cells were differentially susceptible may have been responsible for the reversal of the positive growth trend. Data from another experiment, in which significant differences between growth rates of resistant cv. Ace and susceptible cv. Earlypak-7 were observed for two toxin concentrations, showed an increase in cumulative cell doublings at all toxin concentrations for the duration of the experiment (0 to 136 hours) (Figure 3, Table 10).

A second possibility is that resistant genotype Ace may be intermediate in sensitivity to the host-specific toxin, but has been classified as resistant to the pathogen by field performance. This is supported by the observation that cv. Ace is more sensitive to toxin than cv. Walter by a concentration factor of at least 10-fold, as determined by leaflet bioassays. Although it has been reported that genetic control of sensitivity to the pathogen and the fungal toxin are controlled by the same locus, the published data are not sufficient for a firm conclusion (56). More data are needed.

A third possible explanation for the variable responses to toxin by resistant cv. Ace is that the medium induced a susceptible phenotype in the resistant genotype Ace. This is unlikely, because none of the growth

rates of treated suspension cultures would have been expected to differ from those of comparably treated suspension cultures of the susceptible cultivars. Significant differences between the growth rates of cv. Ace and cv. Earlypak-7 were detected for two toxin concentrations in one experiment.

There were genotype-specific differences in the relative amounts of toxin-induced cell death in suspension cell cultures. The percentage of dead cells increased in proportion to the log of the toxin concentration and the total time of exposure to toxin. A minimum of 30% and a maximum of 75% less cell death occurred in resistant cultures than in susceptible cultures exposed to toxin. In some experiments, resistant genotypes had 35% less cell death at 100-fold higher toxin concentrations than did susceptible cultures at lower toxin concentrations.

It is difficult to compare genotype differences in toxin sensitivity expressed in intact tissue with that expressed in suspension cell cultures. In leaflet bioassays, resistant tomato genotypes consistently tolerated at least 1000-fold higher concentrations of partially-purified toxin preparation than did susceptible. There were always at least 100-fold differences between susceptible and resistant genotypes in suspension cultures. This does not necessarily mean that suspension cultures are less sensitive to toxin than are intact leaflet tissues. There is no obvious way to determine the number of cells affected in a primary or secondary way by the wounding of an intact leaflet and subsequent exposure to a 10 μ l droplet of toxin preparation. It is also difficult, if not impossible, to assess the number of cells in suspension culture affected by direct or secondary contact with toxin molecules, or to determine whether the same number of cells are affected in each

genotype. Several other unknowns also warrant consideration. a) Are toxin effects colligative? b) Is toxin detoxified, or temporarily inactivated and then released in either genotype? c) Are primary and secondary targets of the toxin identical in undifferentiated and differentiated cells? d) Are growth inhibition and cell death in suspension cultures of resistant genotypes a direct consequence of cellular interaction with toxin, or a consequence of toxin metabolism?

Toxin-induced discoloration of suspension cell cultures was a genotype-specific phenomenon expressed only in susceptible tomato genotypes. The degree of discoloration appeared to be correlated with the toxin concentration, the total time of exposure to toxin, and the percentage of toxin-induced cell death. It is probable that the discoloration was caused by oxidation and polymerization of phenolic substances released by toxin-killed cells. Accumulation of oxidized phenolic substances may have caused a cascade effect which enhanced cell death in susceptible genotypes. The absence of toxin-induced discoloration in resistant genotypes suggests that the metabolism differs for resistant and susceptible genotypes.

A second piece of evidence for a difference between resistant and susceptible genotypes in metabolic responses to toxin is the toxin-induced accumulation of fluorescent substances specific to the resistant tomato genotype. The enhancement and possible synthesis of UV-absorbing substances, which accumulated in proportion to the log of the toxin concentration, was observed in supernatant filtrates of resistant cv. Walter, but not in comparably treated susceptible cv. Earlypak-7. Spectrophotometric examination ruled out absorption by residual toxin, medium, or depleted medium. Thin layer chromatographic separation

revealed eleven fluorescent substances, eight of which were readily detected in filtrates of control and toxin-treated suspension cultures. The three fluorescent substances that were detected exclusively in filtrates of toxin-treated suspension cultures may have been present in control filtrates at concentrations that were not detectable in the chromatographic procedure, or they may be substances that were synthesized de novo in response to toxin treatment. Rf values of standards (chlorogenic acid, ferulic acid, p-coumaric acid, caffeic acid, and rishitin solutions) did not correspond to the Rf values of the fluorescent unknowns.

Several speculations concerning the toxin-induced enhancement and possible de novo synthesis of the fluorescent compounds warrant consideration. One obvious possibility is that the substances are stress metabolites, i.e. phytoalexin-like compounds elicited in treated cultures in response to toxin, but possibly present at low concentrations in controls. Cell death in the resistant cv. Walter might not be caused by toxin, but rather by the accumulation of toxic phytoalexin-like compounds. Phytotoxicity of the phytoalexin phaseollin to suspension cultures of Phaseolus vulgaris has been reported (98). Perhaps the fluorescent substances common to the control and toxin-treated suspensions are nonspecific stress metabolites induced in response to manipulation of cells in culture (i.e. pipetting, agitation, anaerobic stress during settled cell volume determinations). The concentration of these may be enhanced in toxin-treated suspension cultures due to an increase in stress on the cultures. The three toxin-induced fluorescent substances which were not detected in the control cultures might be synthesized specifically in response to the toxin.

The fluorescent substances might be secondary metabolites produced by a constitutive enzyme pathway with toxin as a substrate. It is equally plausible to postulate that the toxin is a substrate for an inducible enzyme (or pathway) that is functional at a low constitutive level in untreated cells. The three fluorescent substances specific to the toxin-treated filtrates might be products of a second inducible enzyme that utilizes products of the first pathway as substrates. There is substantial evidence in the biotechnical cell culture literature for the existence of enzymatic pathways in plant cell cultures which process exogenous foreign substrates (4).

Another possibility is that the fluorescent substances are detoxification products of the toxin. Cell death might be attributed to accumulation of detoxification products, or to toxin, if the detoxification mechanism can be flooded. This explanation does not account for the eight fluorescent substances common to the control and toxin-treated suspension cultures, and presumes that the three substances specific to the toxin-treated filtrates are synthesized de novo in response to toxin treatment.

It is premature to accept any of these hypotheses concerning the synthesis and accumulation of the fluorescent compounds. Further studies are underway. Examinations of the responses of resistant cv. Ace and susceptible cv. VFN-Bush are necessary to determine whether this is a phenomenon specific to resistant cultivars, or only to resistant cv. Walter. If resistant cv. Ace is intermediate in sensitivity to the toxin, we might expect induction of some or all of these fluorescent substances, although possibly lower concentrations may accumulate than are present in resistant cv. Walter. A time-course study is underway to

determine when fluorescent substances are first detectable in the resistant cultivar(s), and whether or not they are initially present in the susceptible cultivars. This study is of particular interest because there is a report (19) that tomato callus infected with Phytophthora infestans race 0 produced extractable phenolic substances identical to those extracted from control callus when measured five to seven days after infection. No measurable phenolic substances were detected in infected susceptible callus when extractions were performed ten days after infection. Callus from resistant genotypes did not exhibit comparable loss of extractable phenolic substances at any time during the experiment. A similar phenomenon may occur in suspension cultures of soybean which catabolize phaseollin (98). The last necessary study is a determination of the relationship of the fluorescent compounds to nonspecific stress of the cultures.

Finally, it is possible that the processing of the supernatant filtrates altered or excluded the toxin. The best argument against this suggestion is that the increase in the concentrations of the fluorescent substances, as determined by the increase in UV-absorbance, appeared to be proportional to the log of the toxin concentration. If the toxin were altered or excluded by the extraction procedure, there should have been a change in absorbance proportional to the toxin concentration.

As far as I am aware, all reports of effects of toxins (nonspecific and host-specific) on cell cultures have been conducted with callus cultures. My work demonstrates that suspension cell cultures are appropriate for examination of the physiological responses of cells to host-specific toxins. Preliminary experiments indicated that toxin-treated cells will resume logarithmic growth when rescued from

toxin treatment and resuspended in toxin-free medium. This confirmed the feasibility of using suspension cell cultures for selection of toxin-tolerant or toxin-resistant variants. I have shown that the host-specific toxin from Alternaria alternata f. sp. lycopersici will differentiate between resistant and susceptible genotypes in suspension cultures. Cell culture selections for toxin-tolerance or resistance are feasible in this system.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Carlson, P. S. 1973. Methionine-sulfoximine-resistant mutants of tobacco. *Science* 180:1366-1368.
2. Gengenbach, B. G., C. E. Green, and C. M. Donovan. 1977. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. *Proc. Natl. Acad. Sci.* 74(11):5113-5117.
3. Gressel, J. 1980. "Uses and drawbacks of cell cultures in pesticide research". In Plant Cell Cultures: Results and Perspectives (F. Sala, B. Parisi, R. Cella, and O. Ciferri, eds.), p. 379-388. Elsevier/North-Holland Biomedical Press, Amsterdam.
4. Barz, W., E. Reinhard, and M. H. Zenk, eds. 1977. Plant Tissue Culture and Its Bio-technological Application. Springer-Verlag, New York.
5. Malmberg, R. L., P. J. Koivunemi, and P. S. Carlson. 1980. "Plant cell genetics: stuck between a phenon and its genes". In Plant Cell Cultures: Results and Perspectives (F. Sala, B. Parisi, R. Cella, and O. Ciferri, eds.), p. 15-30. Elsevier/ North-Holland Biomedical Press, Amsterdam.
6. Smith, H. H. 1974. Model systems for somatic cell plant genetics. *Bioscience* 24(5):269-276.
7. Nickell, L. G. 1973. Test-tube approaches to bypass sex. *Hawaiian Planters' Record*. 58(21):293-314.
8. Nickell, L. G., and A. Maretzki. 1972. Developmental and biochemical studies with cultured sugarcane cell suspensions. *Proc. IV IFS: Ferment. Technol. Today*:681-688.
9. King, P. J., K. J. Mansfield, and H. E. Street. 1973. Control of growth and cell division in plant cell suspension cultures. *Can. J. Bot.* 51:1807-1822.
10. Carlson, P. S. 1980. "Blue roses and black tulips: is the new plant genetics only ornamental?" In Linking Research to Crop Production (R. C. Staples and R. J. Kuhr, eds.), p. 63-77. Plenum Pub. Corp., New York.

11. Nickell, L. G., and D. J. Heinz. 1973. "Potential of cell and tissue culture techniques as aids in economic plant improvement". In Genes, Enzymes, and Populations (A. M. Srb, ed.), p. 109-128. Plenum Pub. Corp., New York.
12. Heyns, R. F., A. Rorsch, and R. A. Schilperoort. 1974. Prospects in genetic engineering of plants. Quarterly Rev. Biophys. 7(1):35-73.
13. Henshaw, G. G., K. K. Jha, A. R. Mehta, D. J. Shakeshaft, and H. E. Street. 1966. I. Growth Patterns in batch propagated suspension cultures. Jnl. Expl. Bot. 17(51):362-377.
14. Chaleff, R. S., and P. S. Carlson. 1974. "In vitro selection for mutants of higher plants". In Genetic Manipulations with Plant Material (L. Ledoux, ed.), p. 351-363. Plenum Pub. Corp., New York.
15. Krishnamurthi, M., and J. Tlaskal. 1974. Fiji disease resistant Saccharum officinarum var. Pindar sub-clones from tissue cultures. Proc. Internl. Soc. Sugarcane Tech. XV.:130-137.
16. Heinz, D. J. 1973. "Sugarcane improvement through induced mutations using vegetative propagules and cell culture techniques". In FAO/IAEA Panel Proc. Induced Mutations in Vegetatively Propagated Plants. PL 501/5:53-59.
17. Nickell, L. G. 1977. Crop improvement in sugarcane: studies using in vitro methods. Crop Sci. 17:717-719.
18. Brettell, R. I. S., E. Thomas, and D. S. Ingram. 1980. Reversion of Texas male-sterile cytoplasm maize in culture to give fertile, T-toxin resistant plants. Theor. Appl. Gen. 58:55-58.
19. Warren, R. S., and D. G. Routley. 1970. The use of tissue culture in the study of single gene resistance of tomato to Phytophthora infestans. J. Amer. Soc. Hort. Sci. 95(3):266-269.
20. Helgeson, J. P., J. D. Kemp, G. T. Haberlach, and D. P. Maxwell. 1972. A tissue culture system for studying disease resistance: the black shank disease in tobacco callus cultures. Phytopath. 62:1439-1443.
21. Helgeson, J. P., G. T. Haberlach, and C. D. Upper. 1976. A dominant gene conferring disease resistance to tobacco plants is expressed in tissue cultures. Phytopath. 66:91-96.
22. Haberlach, G. T., A. D. Budde, L. Sequeira, and J. P. Helgeson. 1978. Modification of disease resistance of tobacco callus tissues by cytokinins. Plant Phys. 62:522-525.
23. Behnke, M. 1980. General resistance to late blight of Solanum tuberosum plants regenerated from callus resistant to culture filtrates of Phytophthora infestans. Theor. Appl. Gen.

56:151-152.

24. Bajaj, Y. P. S., and A. W. Saettler. 1970. Effect of halo toxin-containing filtrates of Pseudomonas phaseolicola on the growth of bean callus tissue. Phytopath. 60(7):1065-1067.
25. Matern, U., G. Strobel, and J. Shepard. 1978. Reaction to phytotoxins in a potato population derived from mesophyll protoplasts. Proc. Natl. Acad. Sci. 75(10):4935-4939.
26. Shepard, J. F., D. Bidney, and E. Shabin. 1980. Potato protoplasts in crop improvement. Sci. 208:17-24.
27. Behnke, M. 1979. Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theor. Appl. Gen. 55:69-71.
28. Payne, G. A., and O. C. Yoder. 1978. Effect of the nuclear genome of corn on sensitivity to Helminthosporium maydis race T-toxin and on susceptibility to H. maydis race T. Phytopath. 68:331-337.
29. Nachmias, A., I. Barash, Z. Solel, and G. A. Strobel. 1977. Translocation of mal secco toxin in lemons and its effect on electrolyte leakage, transpiration, and citrus callus growth. Phytoparasitica 5(2):94-103.
30. Ingram, D. S., and N. F. Robertson. 1965. Interaction between Phytophthora infestans and tissue cultures of Solanum tuberosum. J. Gen. Microbiol. 40:431-437.
31. Ingram, D. S. 1967. The expression of R-gene resistance to Phytophthora infestans in tissue cultures of Solanum tuberosum. J. Gen. Microbiol. 49:99-108.
32. _____. 1969. The susceptibility of Brassica callus to infection by Peronospora parasitica. J. Gen. Microbiol. 58:391-401.
33. Keen, N. T., and R. Horsch. 1972. Hydroxyphaseollin production by various soybean tissues: a warning against use of "unnatural" host-parasite systems. Phytopath. 62:439-442.
34. Gengenbach, B. G., and C. E. Green. 1975. Selection of T-cytoplasm maize callus cultures resistant to Helminthosporium maydis race T pathotoxin. Crop Sci. 15:645-649.
35. Brettell, R. I. S., B. V. D. Goddard, and D. S. Ingram. 1979. Selection of Tms-cytoplasm maize tissue cultures resistant to Drechslera maydis T-toxin. Maydica 24:203-213.
36. Gengenbach, G. B., J. A. Connelly, D. R. Pring, and M. F. Conde. 1981. Mitochondrial DNA variation in maize plants regenerated during tissue culture selection. Theor. Appl. Gen. 59:161-167.

37. Kemble, R. J., and J. R. Bedbrook. 1980. Low molecular weight circular and linear DNA in mitochondria from normal and male-sterile Zea mays cytoplasm. *Nature* 284:565-566.
38. Stewart, W. W. 1971. Isolation and proof of structure of wildlife toxin. *Nature* 229:174-178.
39. Brettell, R. I. S., and D. S. Ingram. 1979. Tissue culture in the production of novel disease resistant crop plants. *Biol. Rev.* 54:329-345.
40. Briggs, F. N., and P. F. Knowles. 1967. Introduction to Plant Breeding. Reinhold Pub. Corp., New York.
41. Zilkah, S., P. F. Bocion, and J. Gressel. 1978. Target tissue for napropamide inhibition: effects on green and white callus cultures and seedlings. *Weed Sci.* 26(6):711-713.
42. Zilkah, S., and J. Gressel. 1977. Cell cultures vs. whole plants for measuring phytotoxicity. I. The establishment and growth of callus and suspension cultures; definition of factors affecting toxicity on calli. *Plant Cell Physiol.* 18:641-655.
43. Zilkah, S., P. F. Bocion, and J. Gressel. 1977. Cell cultures vs. whole plants for measuring phytotoxicity. II. Correlations between phytotoxicity in seedlings and calli. *Plant Cell Physiol.* 18:657-670.
44. Zilkah, S., and J. Gressel. 1977. Cell cultures vs. whole plants for measuring phytotoxicity. III. Correlations between phytotoxicities in cell suspension cultures, calli and seedlings. *Plant Cell Physiol.* 18:815-820.
45. _____. 1978. Correlations in phytotoxicity between white and green calli of Rumex obtusifolius, Nicotiana tabacum, and Lycopersicon esculentum. *Pest. Biochem. Physiol.* 9:334-339.
46. Widholm, J. M. 1974. Cultured carrot cell mutants: 5-methyl-tryptophan resistance trait carried from cell to plant and back. *Plant Sci. Let.* 3:323-330.
47. _____. 1977. Selection and characterization of amino acid resistant plant cell cultures. *Crop Sci.* 17:597-600.
48. _____. 1980. "Differential expression of amino acid biosynthetic control isoenzymes in plants and cultured cells". In Plant Cell Cultures: Results and Perspectives (F. Sala, B. Parisi, R. Cella, and O. Ciferri, eds.), p. 157-159. Elsevier/ North-Holland Biomedical Press, Amsterdam.
49. Scheffer, R. P. 1976. "Host-specific toxins in relation to pathogens and disease resistance". In Physiological Plant Pathology. (R. Heitefuss and P. H. Williams, eds.), p. 247-269. Springer-Verlag, New York.

50. Yoder, O. C. 1980. "Toxins in pathogenesis". In Annual Review of Plant Pathology (R. G. Grogan, G. A. Zentmeyer, and G. B. Cowling, eds.), p. 103-129. Annual Reviews, Inc., Palo Alto.
51. Pringle, R. B., and R. P. Scheffer. 1964. "Host-specific plant toxins". In Annual Review of Phytopathology, vol. 2 (J. G. Horsfall and K. F. Baker, eds.), p. 133-156. Annual Reviews, Inc., Palo Alto.
52. Rudolphy, K. 1976. "Non-specific toxins". In Physiological Plant Pathology (R. Heitefuss and P. H. Williams, eds.), p. 270-315. Springer-Verlag, New York.
53. Agrios, G. N. 1969. Plant Pathology. p. 350. Academic Press, New York.
54. Nachmias, A., I. Barash, Z. Solel, and G. A. Strobel. 1977. Purification and characterization of a phytotoxin produced by Phoma tracheiphila, the causal agent of mal secco disease of citrus. Physiol. Plant Path. 10:147-157.
55. Kohmoto, K., R. P. Scheffer, and J. O. Whiteside. 1979. Host-selective toxins from Alternaria citri. Phytopath. 69(6):667-671.
56. Gilchrist, D. G., and R. G. Grogan. 1976. Production and nature of a host-specific toxin from Alternaria alternata f. sp. lycopersici. Phytopath. 66(2):165-171.
57. Nishimura, S., M. Sugihara, K. Kohmoto, and H. Otani. 1978. Two different phases in pathogenicity of the Alternaria pathogen causing black spot disease of Japanese pear. J. Fac. Agric., Tottori Univ. 13:1-10.
58. Kinoshita, T., Y. Renbutsu, I. D. Khan, K. Kohmoto, and S. Nishimura. 1972. Distribution of tenuazonic acid production in the genus Alternaria and its pathological evaluation. Ann. Phytopath. Soc. Japan 38:397-404.
59. Khan, I. D., K. Kohmoto, and S. Nishimura. 1975. Heat-induced resistance of apple leaves against Alternaria mali and its host-specific toxins. Ann. Phytopath. Soc. Japan 41:408-411.
60. Kohmoto, K., I. D. Khan, Y. Renbutsu, T. Taniguchi, and S. Nishimura. 1976. Multiple host-specific toxins of Alternaria mali and their effect on the permeability of host cells. Physiol. Plant Path. 8:141-153.
61. Kohmoto, K., T. Taniguchi, and S. Nishimura. 1977. Correlation between the susceptibility of apple cultivars to Alternaria mali and their sensitivity to AM-toxin I. Ann. Phytopath. Soc. Japan 43:65-68.

62. Otani, H., S. Nishimura, K. Kohmoto, K. Yano, and T. Seno. 1975. Nature of specific susceptibility to Alternaria kikuchiana in Nijisseiki cultivar among Japanese pears (V): role of host-specific toxin in early steps of infection. Ann. Phytopath. Soc. Japan 41:467-476.
63. Otani, H., S. Nishimura, and K. Kohmoto. 1976. Nature of specific susceptibility to Alternaria kikuchiana in Nijisseiki cultivar among Japanese pears. VI. Effect of known plant hormones on sensitivity of pear leaves to host-specific toxin. J. Fac. Agric., Tottori Univ. 11:1-6.
64. Morikawa, M., H. Otani, S. Nishimura, and K. Kohmoto. 1977. Nature of specific susceptibility to Alternaria kikuchiana in Nijisseiki cultivar among Japanese pears. VII. Efflux of cell constituents from pear leaves treated with AK-toxin. J. Fac. Agric., Tottori Univ. 12:1-7.
65. Otani, H., M. Morikawa, S. Nishimura, and K. Kohmoto. 1977. Nature of specific susceptibility to Alternaria kikuchiana in Nijisseiki cultivar among Japanese pears. VIII. Effects of other cations on losses of K^+ after exposure to AK-toxin. Ann. Phytopath. Soc. Japan 43:562-568.
66. Otani, H., S. Nishimura, and K. Kohmoto. 1978. Nature of specific susceptibility to Alternaria kikuchiana in Nijisseiki cultivar among Japanese pears. IX. Effect of other cations on losses of K^+ after exposure to AK-toxin (supplementary report). J. Fac. Agric., Tottori Univ. 13:11-18.
67. Park, P., S. Nishimura, K. Kohmoto, and H. Otani. 1980. Comparative effects of host-specific toxins from four pathotypes of Alternaria alternata on the ultrastructure of host cells. Ann. Phytopath. Soc. Japan. (in press).
68. Grogan, R. G., K. A. Kimble, and I. Misaghi. 1975. A stem canker disease of tomato caused by Alternaria alternata f. sp. lycopersici. Phytopath. 65:880-886.
69. Bottini, A. T., and D. G. Gilchrist. 1981. Phytotoxins I. A 1-amino-dimethylheptadecaptenol from Alternaria alternata f. sp. lycopersici. Tetrahedron Lett. (in press).
70. Bottini, A. T., J. R. Bowen, and D. G. Gilchrist. 1981. Phytotoxins II. Characterization of a phytotoxic fraction from Alternaria alternata f. sp. lycopersici. Tetrahedron Lett. (in press).
71. Kohmoto, K., V. S. Verma, S. Nishimura, M. Tagami, R. P. Scheffer, and R. G. Grogan. 1980. A new outbreak of Alternaria stem canker of tomato in Japan and production of host-specific toxin by the causal pathogen. Ann. Phytopath. Soc. Japan 46(3):387 (Abstr.).

72. McFarland, B. L., and D. G. Gilchrist. 1980. A proposed enzyme specific role for the host-specific toxins of Alternaria alternata f. sp. lycopersici. Phytopath. 71(2):240 (Abstr.).
73. Nishimura, S., K. Kohmoto, and H. Otani. 1979. "The role of host-specific toxins in saprophytic pathogens". In Recognition and Specificity in Plant Host-parasite Interactions (J. M. Daly and I. Uritani, eds.), p. 133-146. University Park Press, Baltimore.
74. Tsuchiya, S., Y. Yoshida, and T. Haniuda. 1967. Studies on the disease resistance in apple. I. Investigaton on the resistance to Alternaria blotch in apple varieties and hybrids. Bull. Hort. Rsh. Sta., Japan, Ser. C 5:9-19.
75. Leary, J. V., and R. M. Endo. 1971. A Fusarium-induced root rot of staked tomatoes. Phytopath. 61(8):900 (Abstr.).
76. Kozaki, I. 1973. Black spot disease resistance in Japanese pear. I. Heredity of the disease resistance. Bull. Hort. Rsh. Stn., Japan, Ser. A 12:17-27.
77. Kasai, T., H. Otani, K. Kohmoto, and S. Nishimura. 1975. Nature of specific susceptibility to Alternaria kikuchiana in Nijisseiki cultivar among Japanese pears. IV. Target tissues of A. kikuchiana toxin and their characteristic responses. J. Fac. Agric., Tottori Univ. 10:6-14.
78. Park, P. 1976. "Initial changes in the ultrastructure of leaf cells of Japanese pear, caused by Alternaria kikuchiana-toxin, tenuazonic acid, and citrinin". In Biochemistry and Cytology of Plant-Parasite Interactions. (K. Tomiyama, J. M. Daly, I. Uritani, H. Ōku, and S. Ouchi, eds.), p. 66-69. Elsevier/North-Holland Biomedical Press, Amsterdam.
79. Ueno, T., T. Nakashima, Y. Hayashi, and H. Iukami. 1975. Structures of AM-toxin I and II, host-specific phytotoxic metabolites produced by Alternaria mali. Agric. Biol. Chem. 39(5):1115-1122.
80. Comstock, J. C., and R. P. Scheffer. 1973. Role of host-selective toxin in colonization of corn leaves by Helminthosporium carbonum. Phytopath. 63(1):24-29.
81. Linsmaier, E. M., and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.
82. Nitsch, J. P. 1969. Experimental androgenesis in Nicotiana. Phytomorph. 19(4):389-404.
83. Widholm, J. M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Tech. 47(4):189-194.

84. Manabe, K., and K. L. Poff. 1978. Purification and characterization of the photoreducible b-type cytochrome from Dictyostelium discoideum. Plant Physiol. 61:961-966.
85. Rieger, R., A. Michaelis, and M. M. Green. 1976. Glossary of Genetics and Cytogenetics, 4th ed. Springer-Verlag, New York.
86. Lucas, G. B. 1965. Diseases of Tobacco, 2nd ed. The Scarecrow Press, New York.
87. Caterino, F. M. 1968. Endopoliploidia e diferenciação. Port. Acta Bio. 11(1):1-218.
88. Witsch, H. V., and A. Flugel. 1952. Über Polyploidieerhöhung im Kurztag bei Kalanchoe blossfeldiana. Zeit. Bot. 40:281-291.
89. Meins, F., Jr., and A. N. Binns. 1978. "Epigenetic clonal variation in the requirement of plant cells for cytokinins". In The Clonal Basis of Development (S. Subtelny and I. M. Sussex, eds.), p. 185-201. Academic Press, New York.
90. Makino, S. 1957. "The chromosome cytology of the ascites tumors of rats with special reference to the concept of the stemline cell". In International Review of Cytology, vol. VI. (G. H. Borne and J. F. Danielli, eds.), p. 25-84. Academic Press, N. Y.
91. Heinz, D. J., and G. W. P. Mee. 1971. Morphologic, cytogenetic, and enzymatic variation in Saccharum species hybrid clones derived from callus tissue. Amer. J. Bot. 58(3):257-262.
92. Belliard, G., F. Vedel, and G. Pelletier. 1979. Mitochondrial recombination in cytoplasmic hybrids of Nicotiana tabacum by protoplast fusion. Nature 281:401-403.
93. Yoder, O. C., and R. P. Scheffer. 1969. Role of toxin in early infections of Helminthosporium victoriae with susceptible and resistant oat tissue. Phytopath. 59:1954-1959.
94. Frear, D. S., and H. R. Swanson. 1975. Metabolism of cisanilide (cis-2,5-dimethyl-1-pyrrolidinecarboxanilide) by excised leaves and cell suspension cultures of carrot and cotton. Pest. Biochem. Physiol. 5:73-80.
95. Oswald, T. H., A. E. Smith, and D. V. Phillips. 1978. Phytotoxicity and detoxification of metribuzin in dark-grown suspension cultures of soybean. Pest. Biochem. Physiol. 8:73-83.
96. Budini, R., D. Tonelli, and S. Girotti. 1980. Analysis of total phenols using the Prussian blue method. J. Agric. Food Chem. 28:1236-1238.
97. Glazener, J. A., and H. D. VanEtten. 1978. Phytotoxicity of phaseollin to, and alteration of phaseollin by, cell suspension cultures of Phaseolus vulgaris. Phytopath. 68:111-117.

98. Christianson, M. L., and M. O. Chiscon. 1978. Use of haploid plants as bioassays for mutagens. *Environ. Health Persp.* 27:77-83.
99. Widholm, J. M. 1972. Cultured Nicotiana tabacum cells with an altered anthranilate synthetase which is less sensitive to feedback inhibition. *Biochim. Biophys. Acta* 261:52-58.
100. Matthews, B. F., and J. M. Widholm. 1979. Enzyme expression in soybean cotyledon, callus and cell suspension culture. *Can. J. Bot.* 57(4):299-304.
101. Pring, D. R., C. S. Levings, III, W. W. L. Hu, and D. H. Timothy. 1977. Unique DNA associated with mitochondria in the "S"-type cytoplasm of male-sterile maize. *Proc. Natl. Acad. Sci.* 74(7):2904-2908.
102. Kende, H., and B. Baumgartner. 1974. Regulation of aging in flowers of Ipomoea tricolor by ethylene. *Planta* 116:279-289.
103. Shaffer, B. M. 1975. Secretion of cyclic AMP induced by cyclic AMP in the cellular slime mold Dictyostelium discoideum. *Nature* 255:549-552.
104. Cheng, T-Y. 1972. Induction of indoleacetic acid synthetases in tobacco pith explants. *Plant Physiol.* 50:723-727.
105. Steiner, G. W., and R. S. Byther. 1971. Partial characterization and use of a host-specific toxin from Helminthosporium sacchari on sugarcane. *Phytopath.* 61(6):691-695.
106. Steiner, G. W., and G. A. Strobel. 1971. Helminthosporoside, a host-specific toxin from Helminthosporium sacchari. *J. Biol. Chem.* 246(13):4350-4357.
107. Strobel, G. A. 1973. The helminthosporoside-binding protein of sugarcane. *J. Biol. Chem.* 248(4):1321-1328.
108. Livingston, R. S., and R. P. Scheffer. 1981. Isolation and characterization of host-selective toxin from Helminthosporium sacchari. *J. Biol. Chem.* 256(4):1705-1710.
109. Byther, R. S., and G. W. Steiner. 1972. Use of helminthosporoside to select sugarcane seedlings resistant to eye-spot disease. *Phytopath.* 62:466-470.
110. Levings, C. S., III, B. D. Kim, D. R. Pring, M. F. Conde, R. J. Mans, J. R. Laughnan, and S. J. Gabay-Laughnan. 1980. Cytoplasmic reversion of cms-S in maize: association with a transpositional event. *Science* 209:1021-1023.
111. Yoder, O. C., and V. E. Gracen. 1975. Segregation of pathogenicity types and host-specific toxin production in progenies of crosses between races T and O of Helminthosporium maydis

- (Cocchliobolus maydis). Phytopath. 65:273-276.
112. Yoder, O. C., G. A. Payne, P. Gregory, and V. E. Gracen. 1977. Bioassays for detection and quantification of Helminthosporium maydis race T-toxin: a comparison. Physiol. Plant Path. 10:237-245.
 113. Edgerton, C. W. 1958. Sugarcane and Its Diseases. Louisiana State University Press, Baton Rouge.
 114. Dean, J. L., and J. D. Miller. 1975. Field screening of sugarcane for eye spot resistance. Phytopath. 65(9):955-958.
 115. Ames, B. N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mut. Res. 31:347-364.
 116. Patel, P. N., and J. C. Walker. 1963. Changes in free amino acid and amide content of resistant and susceptible beans after infection with the halo blight organism. Phytopath. 53(5):522-528.